STUDY OF EXPRESSION OF p16INORAL SQUAMOUS CELL CARCINOMA, POTENTIALLY MALIGNANT DISORDERS ANDNORMAL MUCOSA

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CERTIFICATE

This is to certify that this dissertation titled **"STUDY OF EXPRESSION OF p16 IN ORAL SQUAMOUS CELL CARCINOMA, POTENTIALLY MALIGNANT DISORDERS AND NORMAL MUCOSA"** is a bonafide dissertation performed done by **R.SUDHARSAN** under our guidance during his postgraduate study period between 2010-2013.

This dissertation is submitted to **THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY,** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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ABSTRACT

BACKGROUND:

It has been hypothesized that inactivation of p16 plays a vital role in evolution of potentially malignant disorders [Epithelial Dysplasia, Oral SubmucousFibrosis (OSF)] and in Oral Squamous Cell Carcinoma (OSCC). We wanted to ascertain the role of p16 in OSF, epithelial dysplasia and OSCC as an early marker for malignant transformation and its liability in OSCC.

AIM:

To study the expression of p16 in normal mucosa (Group I), OSF (Group II), epithelial dysplasia (Group III) and OSCC (Group IV).

MATERIALS AND METHODS:

A total sample size of 70 which includes 10 controls of Group I, 20 cases of Group II, 20 cases of Group III, 20 cases of Group IV were studied for p16 expression by immunohistochemistry.We have employed p16 monoclonal antibody of clone G175-405 in our study. The positive control sample of our study is squamous cell carcinoma of uterine cervix.

RESULTS:

Out of 70 samples 16 cases (22.9%) of cases were p16 positive which includes five in group I, seven in group II, one in group III, three in group IV. Out of sixteen p16 positive samples four cases showed cytoplasmic staining (12,1 in Group I,II,IV respectively) , eleven cases showed nuclear and cytoplasmic staining (3,5,1,2 in Group I,II,III,IV respectively) and one showed cytoplasmic and membrane staining.

CONCLUSION:

In this study we addressed the association particularly between p16 and OSF, epithelial dysplasia and OSCC. The results of this study with respect to OSF data, highlights that p16 could play a role in malignant transformation of OSF and we hypothesize that it could be associated with HPV.Further studies should ascertain the HPV status of the cases to be included, with a larger sample size to establish and understand if p16 expression could have a role in oral potentially malignant lesions and OSCC.

KEYWORDS - p16, OSF, OSCC, Epithelial Dysplasia

CONTENTS

INTRODUCTION

Oral squamous cell carcinoma (OSCC), is the sixth most common cancer worldwide and the third most common form of cancer in the developing countries. Squamous cell carcinoma occurs due to multiple genetic changes leading to formation of either abnormal proteins or altered amount of normal proteins[1].

Clinical OSCC is often preceded by stepwise transition from potentially malignant states like leukoplakia and Oral Submucous Fibrosis (OSF) to the metastatic tumour phenotype. A variety of alterations accumulate to potentiate this transition to malignancy[2].

Leukoplakia, the most common potentially malignant lesion of the oral mucosa is defined by WHO as "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease". The malignant transformation rate for leukoplakia ranges from 5-20% and is particularly correlated with the degree of dysplasia[3]. The transition from normal oral epithelium to oral dysplasia and cancer results from accumulated genetic and epigenetic alterations[4]. The grading of epithelial dysplasia remains subjective as it relies on cellular atypia and architectural disturbances.

OSF is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat[5].

Given this aggressive nature of the potentially malignant lesions**,** identification of a suitable biomarker is imperative for timely diagnosis, prognosis and treatment[6]. Mutations in tumour suppressor genes namely p53, pRb, p16 and pro-apoptotic genes namely bcl2, bax have been variously attributed to the development and transformation of precancer to cancer. It has been found that inactivation of p16 occurs early in the development of OSCC[7].

Homozygous deletion, point mutation, loss of heterozygosity and the more common aberrant methylation are the frequently reported alterations in the p16 gene[8]. p16 is a cyclin dependent kinase inhibitor(CKI), a tumour suppressor gene, and is the second commonly affected gene next to p53 in OSCC. The main function of p16 is to control the phosphorylation of retinoblastoma (Rb) gene and block the progression of cell cycle.

Human papilloma virus (HPV) has been reported to initiate carcinogenesis in cervical cancers and OSCC. HPV causes oral carcinogenesis by acting on viral oncoproteins E6 and E7. P16 inactivates RB protein[67]. Similarly overexpression of p16 in cervical cancer is due to functional inactivation of Rb by HPV E7 oncoprotein. Thereby, it clearly shows that HPV causes inactivation of p16 pathway which leads to malignant transformation and carcinogenesis. There is a strong association between HPV presence and p16 in certain neoplasms.

In this study, we wanted to ascertain the immunohistochemical expression of p16 in leukoplakia, OSF, OSCC and compare it with its expression in normal mucosa. We also included cases which were suggestive of HPV infection histopathologically, to ascertain its association.

AIM AND OBJECTIVES

AIM:

To assess the expression of p16 in normal oral mucosa, potentially malignant disorders (Leukoplakia, OSF) and OSCC.

OBJECTIVES:

1. To study the expression of p16 in formalin fixed paraffin embedded tissue specimens of normal oral mucosa by immunohistochemistry (IHC).
2. To study the expression of p16 in formalin fixed paraffin embedded tissue specimens of epithelial dysplasia (Leukoplakia) by IHC.
3. To study the expression of p16 in formalin fixed paraffin embedded tissue specimens of OSF by IHC.
4. To study the expression of p16 in formalin fixed paraffin embedded tissue specimens of OSCC by IHC.
5. To compare the expression of p16 between normal oral mucosa, Leukoplakia, OSF and OSCC.

STUDY SETTING:

The study was conducted in Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using formalin fixed paraffin embedded tissues. It is a retrospective study done to evaluate the expression of p16 in normal mucosa, OSF, leukoplakia, OSCC and normal mucosa using immunohistochemistry in formalin fixed, paraffin embedded tissue

specimens. The approval from Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai was obtained. (ANNEXURE I)

HYPOTHESIS: (NULL)

There is no difference in expression of p16 in potentially malignant disorders (Leukoplakia, OSF), OSCC when compared to normal oral mucosa.

STUDY SUBJECTS:

The study material comprised of 70 formalin fixed, paraffin embedded tissue specimens. The samples were divided into 4 groups namely: Group I, Group II, Group III and Group IV.

Group I: 10 normal mucosa tissue specimens.

Group II: 20 histopathologically confirmed OSF fibrosis tissue specimens.

Group III: 20 histopathologically confirmed epithelial dysplasia (Leukoplakia) tissue specimens.

Group IV: 20 histopathologically confirmed OSCC tissue specimens.

METHODOLOGY

1. Tissue samples of Normal mucosa (n=10), OSF (n=20), Leukoplakia (n=20) and OSCC (n=20) were taken from the patients and from the archival blocks.
2. A detailed case history including age, gender and occupation, past medical history & past dental history, history of drugs and trauma were recorded.
3. General examination and intra oral examination was done.
4. Biopsy was done from the lesion site. Normal mucosa was taken when the patients were undergoing minor surgery for impacted teeth cases.
5. The tissue biopsied was immediately transferred to 10 % buffered formalin.
6. After adequate fixation, tissues were embedded in paraffin.
7. From the paraffin embedded blocks 4 microns thick, sections were cut and used for routine Hematoxylin and Eosin (H&E) staining and immunohistochemical (IHC) staining.
8. Tissue sections of squamous cell carcinoma of cervix were used as positive controls for p16 positivity.

HEMATOXYLIN & EOSIN (H&E) STAINING:

REAGENTS:

Harris's hematoxylin

1% acid alcohol

Eosin

PROCEDURE:

The slides were dewaxed in xylene and hydrated through graded alcohol to water. The sections on the slides were flooded with Harris's hematoxylin for 5 minutes. The slides were washed in running tap water for 5 minutes. The slides were differentiated in 1% acid alcohol for 5 minutes.

The slides were washed well in running tap water for 5 minutes. The tissue sections on the slides were then stained in eosin for 30 seconds. The slides were washed in running tap water for 1 minute. The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).

IHC:

ARMAMENTARIUM:

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminium foil
- Micro-pipettes
- Toothed forceps

- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

REAGENTS USED:

- Concentrated HCl
- Laxbro solution
- APES (3 amino propyl tri ethoxy silane)
- Acetone
- Citrate buffer
- Phosphate Buffer Saline (PBS)
- 3% H_2O_2
- Deionized distilled water
- Absolute alcohol
- Xylene

ANTIBODIES USED:

1. Primary antibody – p16 monoclonal antibody, BIOGENEX^{TM} Clone: G175 – 405

   (FIGURE:1)
2. Secondary antibody – Biogenex-super sensitive IHC detection system kit^{TM} (Poly Horse Radish Peroxidase - pretitrated anti-species

immunoglobulins labelled with enzyme polymer, super enhancer reagent, antimouse monoclonal negative control serum, and liquid DAB Diamino-benzidine-chromogen) (FIGURE: 1)

IHC PROCEDURE:

PRETREATMENT OF THE SLIDES:

- The slides were first washed in tap water for few minutes.
- The slides were then soaked in detergent solution for 1 hour.
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water.
- The slides were washed in autoclaved distilled water.
- The slides were immersed in 1 N HCL (100 ml HCl in 900 ml distilled water) overnight.
- The following day slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees Centigrade.

APES (3 Amino propyl tri ethoxy silane) coating:

Slides first dipped in couplin jar containing acetone for 2 minutes

↓

Dipped in APES for 5 minutes

↓

Dipped in two changes of distilled water for 2 minutes each

↓

Slides left to dry

PREPARATION OF PARAFFIN SECTIONS:

After the slides were dry, tissue section of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labelled positive (P) and the other negative (N).

PROCEDURE:

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Slides were then treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining. The slides were then transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. The slides were dipped in 3 changes of distilled water for 5 minutes each. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The tissues were incubated in protein blocking serum for one hour in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The primary antibody, p16 monoclonal antibody was added to P tissue on the slide and then to the N, PBS was added. The slides were incubated for one hour. Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. Then a drop of ENVISION^{TM} horseradish peroxidase was added on both the sections and the slides were incubated for 30 minutes. Later slides were washed in

three changes of cold PBS for 5 minutes in each. The sections were washed in 3 changes of cold PBS for 5 minutes in each. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of DAB was added to the sections for 5 minutes. Slides were then washed in distilled water to remove excess chromogen and counter stained with hematoxylin. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with DPX. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

IHC PROCEDURE FLOW CHART:

APES coated slides with 2 paraffin embedded tissues

↓

Placed in xylene thrice (5 minutes each)

↓

Placed in 100% isopropanol (5 minutes)

↓

Placed in 70% isopropanol (5 minutes)

↓

Washed in distilled water thrice (5 minutes each)

↓

Placed in 3% hydrogen peroxide (20 minutes)

↓

Kept in citrate buffer at pH 6 and autoclaved for antigen retrieval and bench cooled for 40 minutes

↓

Washed in distilled water thrice (5 minutes)

↓

Protein blocking serum added and incubated for one hour

↓

Primary antibody added to the specimen and incubated for one hour

↓

Washed in PBS thrice (5 minutes each)

↓

Secondary antibody added and incubated in an enclosed hydrated container (30 minutes)

↓

Washed in PBS thrice (5 minutes each)

↓

HRP secondary reagent added and incubated (30 minutes)

↓

Washed in PBS thrice (5 minutes each)

↓

DAB added and incubated in an enclosed hydrated container (5 minutes)

↓

Washed in PBS thrice (5 minutes each)

↓

Stained with hematoxylin (20 seconds)

↓

Washed in tap water

↓

Placed in 70% isopropanol (1minute)

↓

Placed in 90% isopropanol (1minute)

↓

Placed in 100% isopropanol (1 minute)

↓

Placed in xylene (1 dip)

↓

Slides were mounted using DPX

↓

Slides were observed under the Light Microscope and graded

Criteria for detecting the expression of p16:

1. H&E sections:
    - The H&E stained sections were thoroughly examined.
    - Dysplastic lesions were categorised as mild dysplasia, moderate dysplasia and severe dysplasia. The koilocytic changes were also noted in dysplastic sections.
    - Oral squamous cell carcinoma was graded as well differentiated, moderately differentiated, poorly differentiated.
2. Corresponding sections as examined by H&E were stained by IHC to detect p16 expression.
3. To all these sections the p16 antibody and the phosphate buffer saline was added based on the IHC protocol for the p16 antibody.

4. For IHC

- The positive control used for IHC was squamous cell carcinoma of cervix.
- The stained slides were screened, examined systematically for p16 expression in the nucleus, cytoplasm and also for membrane staining.
- Positive cells were counted in the basal, suprabasal layers of normal mucosa.
- Positive cells were counted in the basal, suprabasal layers in dysplasia, basal and suprabasal layers in oral submucous fibrosis, in the invasive front of oral squamous cell carcinoma.
- Percentage of positive cells were also counted in each case and it was categorised as

  0 = negative; 1+ = 1% to 25% of cells positive; 2+ = 26% to 50%; 3+ = 51% to 75%; 4+ = 76% to 100%.
- Connective tissue was also examined in all the lesions.
- Nuclear staining was expressed as percentage of cells and the positivity were noted as mean labelling index (MLI) and cytoplasmic staining was also recorded.
- 0, 1+, 2+, 3+, 4+ staining with reference to the positive control was observed with reference to nuclear and cytoplasmic staining grading.
- The MLI for all the positive groups were calculated using the formula:

$$\frac{\textbf{Number of positive cells}}{\textbf{Total number of cells}} \textbf{ X 100}$$

STATISTICAL ANALYSIS:

Statistical analysis was done using SPSS^{TM} version 17. Proportion of p16 staining was compared between and within the study groups using 'chi square' test. 't' test and 'oneway ANOVA' was done for continuous variables. A p value of 0.05 was considered statistically significant.

SAMPLE SIZE:

To study the difference in expression of p16 between normal mucosa, OSF, epithelial dysplasia, OSCC a total of 70 cases were studied (10 normal mucosa, 20 OSF, 20 epithelial dysplasia and 20 OSCC) with a power of 80% and at 5% significance level.

REVIEW OF LITERATURE

CELL CYCLE:

The basic cell cycle is divided into four stages: G1, S, G2, and M. G1 is the gap phase during which cells prepare for the process of DNA replication. It is during the 'G1' phase that the cell integrates mitogenic and growth inhibitory signals and then decides to pause, or exit the cell cycle. An important checkpoint in 'G1' has been identified in which the cell becomes committed to DNA replication and completes a cycle. 'S' phase is defined as the stage in which DNA synthesis occurs. 'G2' is the second gap phase during which the cell prepares for the process of division. M stands for mitosis, the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. In addition to G1, S, G2, and M, the term G_0 is used to describe cells that have exited the cell cycle and become quiescent[9].

CDK INHIBITORS: INK4 and Cip/Kip FAMILY:

Cyclin Dependent Kinase inhibitor proteins serve as an important mechanism during cell cycle, by preventing the premature entry of the cell into the S phase. This is brought about by binding of cyclin dependent kinase inhibitors (CKIs) to cyclin dependent kinases (CDKs) and thus inactivating CDKs activity, leading to a negative regulation of the cell cycle. The CDK activity is regulated by two families of inhibitors. The first family of CKIs are the Inhibitors of CDK4 (INK4) proteins which include INK4A, INK4B, INK4C and INK4D. The second family includes the Cip and Kip family which are CDK inhibitory protein/ Kinase inhibitor proteins (Cip/Kip) family in mammals. This family is composed of

p21Cip1, p27Kip1 and p57Kip2. The CKIs function as a break for the premature entry of the cell to the S phase by inhibiting the CDKs[10].

INK4/ARF LOCUS AND p16INK4a:

The INK4 gene family encodes for p16INK4a, p15INK4B, p18INK4C, and p19INK4D, all of which bind to CDK4 and CDK6 and inhibit their kinase activities by interfering with their association with D-type cyclins. Specific polypeptide inhibitors of CDK4 and CDK6, so-called the INK4 proteins (inhibitors of CDK4), can directly block cyclin D-dependent kinase activity and cause G1 phase arrest.

The INK4/ARF locus encodes three tumour suppressors, p15INK4B, p16INK4a and p19ARF. p16INK4a has been given different names (CDKN2A p16INK4a, CDK4I, MTS1, and p16). The gene is composed of 3 exons, with one alternatively spliced exon (El-f.). In humans it is situated on chromosome 9p21. pl6CDKN2A protein encodes a 156 amino acid, 16kD cell cycle inhibitor protein, which normally blocks abnormal cell growth and proliferation by binding to complexes of cyclin-dependent kinases 4 and 6, and cyclin D. p16INK4a and p19ARF are transcribed using alternative exons 1α and 1β and spliced onto the same exons but in different open reading frames. Despite overlapping coding regions, p16INK4a and p19ARF have unrelated amino acid sequences and distinct functions. Both p16INK4a and p19ARF are able to inhibit the cell cycle progression and are therefore referred to as tumour suppressors. p16INK4a regulates the cell cycle by inhibiting the CDK4 or CDK6 cyclin dependent kinases. Inhibition of CDK4 or CDK6 prevents the phosphorylation of pRb

proteins and progression into the S phase. pRb is a key gatekeeper in cell cycle transition. In normal cycling cells, entry to the S-phase from G1 phase is related to the functional inactivation of pRb by phosphorylation by cyclin dependent kinases[11].

INK4A/ARF locus encodes two distinct products. Exon1α encodes part of p16INK4a protein which is a cyclin dependent kinase inhibitor. Exon1β is spliced onto an acceptor site common to 1α but encodes a nonhomologous protein called p19ARF, in an alternative reading frame. p16INK4a inhibits the CDK 4 and 6 thus causing inhibition of E2F transcription factors. INK4A/ARF locus encodes two distinct proteins functioning upstream of both Rb and p53 pathways in the cell. The INK4A/ARF locus represents a unique phenomenon by encoding two distinct tumour suppressor proteins inhibiting the cell cycle progression in a different manner. p16INK4a acts by inhibiting CDK4 and 6 and keeping pRb active while p19ARF leads to stabilization of p53. Functions of these proteins upstream of both pRb and p53 make this locus a 'keystone gene' in cell cycle[12].

p16 AND CARCINOGENESIS:

The CDKN2A gene locus or the INK4a/ARF locus encodes two protein products; the p16/INK4a CKI, which blocks cyclin D/CDK2-mediated phosphorylation of Rb, keeping the Rb checkpoint in place. The second gene product, p14ARF, activates the p53 pathway by inhibiting MDM2 and preventing destruction of p53. Both protein products function as tumour suppressors and thus mutation or silencing of p16 locus impacts both the Rb and p53 pathways. p16 is crucial for the induction of senescence. Mutations at this locus have been detected

in oral cancer. In some tumours, such as cervical cancer, p16INK4a is frequently silenced by hypermethylation of the gene. The other CKIs also function as tumour suppressors and are frequently mutated or otherwise silenced in many human malignancies, including familial melanomas, sporadic pancreatic adenocarcinomas, and squamous cell carcinomas of the esophagus[13].

Hypophosphorylated Rb in complex with the E2F transcription factors binds to DNA, recruits chromatin-remodeling factors (histone deacetylases and histone methyl transferases), and inhibits transcription of genes whose products are required for the S phase of the cell cycle. When Rb is phosphorylated by the cyclin D–CDK4, cyclin D–CDK6 and cyclin E–CDK2 complexes, it releases E2F. The latter then activates transcription of S-phase genes. The phosphorylation of Rb is inhibited by CKIs, because they inactivate cyclin-CDK complexes. Virtually all cancer cells show dysregulation of the G_1-S checkpoint as a result of mutation in one of four genes that regulate the phosphorylation of Rb; these genes are Rb1, CDK4, the genes encoding cyclin D proteins, and CDKN2A(p16)[14].

Tsantoulis PK *et al* (2007) emphasized the importance of cancer transforming from the transitional precursor lesions. They also elucidated the genetic implications of the molecular mediators in cancer which includes cyclin dependent kinase inhibitors, TP53 and Rb1 and oncogenes like the cyclin family. They also summarised the tumorigenic effects of HPV and EBV[15].

Malumbres M *et al* (2005) reviewed the role of cyclin dependent kinases in cell proliferation. Based on their genetic study using mice, they proposed that CDK4 and CDK6 are not essential for organogenesis but are involved in the

generation of endocrine and hematopoietic cells. They found that CDK2 is important for the first meiotic division of male and female germ cells. These suggested that CDKs are important for the regulation of cell cycle and that they can be utilised for therapeutic strategies in cancer cells[16].

p16 GENE REGULATION AND INACTIVATION:

Serrano M *et al* (1993) stated that the cell division of eukaryotic cells is regulated by a family of protein kinases which were latter classified as cyclin dependent kinases. The sequential activation of individual members of the family and their consequential phosphorylation of critical substrates promotes orderly progression through cell cycle. They concluded that p16INK4A acted at both the upstream and downstream function in the cell cycle of Rb to form a negative feedback loop which regulates the ability of Rb to prevent cell proliferation[17].

Tam SW *et al* (1994) studied the differential expression of p16 in 9 cases of 3 primary tissues namely; epithelium, muscles and nervous tissue wherein they found the differential expression of CKIs which played a key role in cell cycle regulation. They found that there was increase in levels of p16 along with functional inactivation of the retinoblastoma gene product. They also found that p16INK4a expression varies during the cell cycle peak during S phase. Their results showed a functional relationship between p16INK4A and the retinoblastoma gene product which indicates that p16INK4A is required for CDK4 inhibition only at the G1-S transition at the time when CDK4 kinase activity is not necessary[18].

Mao L *et al* (1995) used cell lines derived from head and neck cancers, HeLA cell lines and normal lymphoblastic cells to investigate the presence of alternative promoter or initiator site showing methylation. p16INK4A AND p15INK4B were localized to chromosome 9p21, and pl6 was subsequently found to be mutated in familial melanoma and deleted in a wide variety of sporadic cancers. This finding emphasized the *denovo* methylation of 5' CpG island resulting in transcriptional block of full length p16 in many neoplasms[19].

Stone S *et al* (1995) studied the genomic structure and transcriptional regulation of p16 gene in humans by using genomic cloning, cDNA clones, in cell lines, lymphocytes by flow cytometry. From this two types of promoters α and β were found and were of similar size. These results showed p16 gene is complex with α and β promoters with different coding potential. They concluded that genetic evidence of p16 and Rb are members of growth regulatory pathway often inactivated leading to tumour progression. They also stated that if the role of β transcript is regulation of cell growth negatively, it could probably be another pathway that could be independent of p16 and Rb. They also used molecular genetic techniques to explore the role of p16 in normal development and cancer. They found that p16 derived mRNAs are probably generated from separate promoters and transcription from one of the promoters appears to be regulated, atleast in part, by the retinoblastoma gene product[20].

Beausejour CM *et al* (2003) proposed that telomere erosion and subsequent dysfunction limits the proliferation of normal human cells by a process termed replicative senescence. Replicative senescence is thought to suppress

tumorigenesis by establishing an essentially irreversible growth arrest that requires activities of the p53 and pRb tumour suppressor proteins. They showed that, depending on expression of the pRb regulator p16, replicative senescence is not necessarily irreversible. Expression of telomerase did not reverse the senescence arrest. Their results indicated that the senescence response to telomere dysfunction is reversible and is maintained primarily by p53. However, p16 provides a dominant second barrier to the unlimited growth of human cells[21].

Ohtani N *et al* (2004) summarised the mechanisms involved in the senescence of p16 which leads to tumour suppression. They summarised the importance of telomere shortening which leads to senescence and telomere independent mechanisms which involves oxidative stress and inactivation of p16-Rb pathway which leads to cancer[22].

p16 IN OTHER NEOPLASMS:

Herman J *et al* (1995) studied the promoter methylation of p16 in breast cancer, prostate cancer, renal cancer and colon cancer. They found aberrant *denovo* methylation of p16 in all the cancer cell lines. They concluded that p16 promoter methylation plays an important diagnostic and prognostic factor for all the common human cancers[23].

Foulkes W *et al* (1997) reviewed the significance of p16 in different human neoplasms. They emphasized the presence of point mutations in familial atypical multiple mole/melanoma, pancreatic cancer and somatic mutations which includes homozygous deletions and CpG island methylations in different

carcinomas. They also signified the low frequency of p16 mutations in common tumours such as ovarian cancer, breast cancer and colon cancer[24].

p16 IN VASCULAR SMOOTH MUSCLE CELLS:

Tanner F *et al* (2000) studied the expression of p27, p21 and p16 in vascular smooth muscle cells. Their results showed that p27 and p21 inhibits the proliferation of vascular smooth muscle cells while p16 has no interference in it. This suggested that p16 has no influence in growth of vascular smooth muscle cells[25].

p16 IN OESOPHAGEAL CANCERS:

Mathew R *et al* (2001) studied the immunohistochemical expression of p5, pRb, p21, p16 to analyse the prognostic significance of these proteins and their alterations in the molecular basis of oesophageal cancers. They analysed 100 oesophageal squamous cell carcinomas and found overexpression of p53, MDM2 and cyclin D1 proteins in 73, 42 and 67% of the cases, respectively, and loss of expression of p21, p16 and pRb in 36, 45 and 75% of the cases, respectively. From the results, they concluded that p16 cannot be used as a specific marker for oesophageal cancer[26].

p16 IN CERVICAL CANCERS:

Volgareva G *et al* (2004) studied the immunohistochemical expression of p16INK4a to determine the specificity of it in dysplastic and neoplastic cervical epithelium. They analysed 194 samples in total which included vaginal smears and biopsies. They found that p16INK4a is overexpressed in dysplastic and

neoplastic cervical epithelium and had a negative expression in normal tissues. Their results suggested that p16INK4a is reliable for early diagnosis of cervical neoplasm[27].

p16 IN ODONTOGENIC CYSTS:

Artese L *et al* (2008) studied the expression of p16 in different odontogenic cysts to correlate the clinical pathology of these cysts. Their results showed overexpression of p16 in radicular or follicular cysts and loss of expression in keratocystic odontogenic tumours. These differences suggested that aggressive potential of keratocystic odontogenic tumour may be related to decrease in p16 expression[28].

LEUKOPLAKIA:

The term leukoplakia was coined by Schwimmer in 1877 to define a white lesion of the tongue. According to WHO, leukoplakia is a white patch or plaque that cannot be characterized clinically or pathologically as any other disease. Leukoplakia occurs mostly in middle-aged and older men. The most common sites are the buccal mucosa, alveolar mucosa, and lower lip. The lesions in the floor of mouth, lateral tongue, and lower lip also shows dysplastic or malignant changes. Early leukoplakia appears as a well defined slightly elevated grayish-white plaque or gradually blends into the surrounding normal mucosa. On progression of the lesion, it becomes thicker and whiter, and develops a leathery appearance with surface fissures (homogeneous or thick leukoplakia). Leukoplakias which develop surface irregularities are described as granular or nodular leukoplakias. Lesions developing a papillary surface are known as verrucous or verruciform leukoplakia.

Proliferative verrucous leukoplakia (PVL), is characterized by widespread, multiple sites of involvement, usually in patients without known risk factors[29].

MALIGNANT TRANSFORMATION OF EPITHELIAL DYSPLASIA:

Hall G *et al* (2008) studied the malignant transformation potential of epithelial dysplasia in 284 biopsies. They used pyrosequencing assays along with methylation specific PCR. They analysed 24 non transforming cases, 14 transforming cases and 38 samples of epithelial dysplasia. Their results showed 57 of the cases which transformed to oral squamous cell carcinoma showed p16 promoter methylation and 2% of the cases which does not undergo malignant transformation were negative for p16 methylation. From their results they proposed that p16 can be used as a predictor for malignant transformation[30].

Wang Z *et al* (2009) studied the pathways of oral epithelial dysplasia transforming into a malignancy. They used two dimensional electrophoresis to study the proteins involved in the malignant transformation. From their results they found that varying levels of differentially expressed proteins were responsible for the malignant transformation. In particular three homologs of PA28 are significant in malignant transformation[31].

p16 IN EPITHELIAL DYSPLASIA:

Papadimitrakopoulou V *et al* (1997) found that the overexpression of the protein products of genes associated with the cell cycle tumour protein 53 (p53), CKI 2A and antigen identified by monoclonal antibody Ki-67 (Ki-67). They studied the overexpression of these factors in 76 leukoplakia biopsy specimens

and interpretable staining in 74 biopsy specimens and by direct sequencing analysis they found loss of heterozygosity in one specimen out of 10 biopsy specimens. The staining in dysplastic are mostly nuclear and cytoplasmic staining is not significant. They proposed that the loss of expression may be due to 3 possible mechanisms. One mechanism is the use of only one marker to characterize the chromosomal region was not sufficient to detect a small deletion which may lead to the loss of protein expression. Second possibility is that normal contaminating DNA may cause retention pattern in cases of homozygous deletion. Third mechanism may be due to DNA methylation of p16INK4a causing loss of protein expression[7].

Bradley K *et al* (2006) showed immunohistochemical overexpression of p16 in dysplastic lesions of the oral cavity. The frequent occurrence of p16 inactivation during early carcinogenesis has led to its investigation as a surrogate marker for dysplasia. They studied 119 biopsy specimens representing various oral cavity sites and degrees of dysplasia; showed 61/119 (51%) cases showed no p16 immunoreactivity, including 12/33 (36%) cases of non dysplastic mucosa, 11/28 (39%) cases of mild dysplasia, and 38/58 (66%) cases of moderate/severe dysplasia. The remaining cases showed p16 expression limited to the basal and suprabasal nuclei and generally confined to the lower one-third of the epithelium. Based on the results which showed decreased expression of p16 in dysplastic lesions they concluded that p16 immunohistochemistry is not helpful in differentiating dysplastic from non dysplastic mucosa. They also concluded that downregulation of p16 contributed to cellular proliferation, resulting locally in a more advanced tumour. The decreased expression of p16 may be due to inactivation of p16[32].

Angiero F *et al* (2008) studied the expression of the cell-cycle proteins p16 and p53 in the dysplastic epithelium, in association with Ki-67 which may represent significant markers to recognize evolution of precancerous disease in the oral cavity and to improve identification of the degree of dysplasia**.** The nuclear expression of p53 and Ki- 67 and nuclear and/or cytoplasmic expression of p16 protein was examined in 54 biopsy specimens from the oral cavity obtained over a period of 3 years. Results showed p53 and p16 expression respectively in 81.8% and 54.5% of cases, while Ki-67 was elevated in all the cases. The expression of the cell-cycle proteins p16 and p53 in the dysplastic epithelium, in association with Ki-67, may represent significant markers to recognize evolution of precancerous disease in the oral cavity and to improve identification of the degree of dysplasia[33].

Liu M *et al* (2010) reported that that promoter hypermethylation of key genes in critical pathways is common in head and neck squamous cell cancers (HNSCC), as well as in serum and saliva of patients with such cancers. p16INK4a is critical at the G1-S transition of the cell cycle, being responsible for maintaining Rb protein in its nonphosphorylated state. From the analysis of methylation specific polymerase chain reaction (PCR) in 111 patients who presented with oral leukoplakia they found that multiple epigenetic abnormalities have already occurred in oral premalignant lesions similar to early genetic alterations. They concluded that p16 promoter hypermethylation is present in most of the premalignant lesions and cancers as well[34].

HPV IN EPITHELIAL DYSPLASIA:

Fregonesi PA *et al* (2003) studied about the presence of HPV in epithelial dysplasia, hyperplasia and squamous cell carcinoma by insitu hybridisation and immunohistochemistry in 46 oral biopsy specimens which were oral hyperplasias, Oral squamous papilloma, oral premalignant lesions, oral squamous cell carcinoma respectively. From their results they found that 18 of the 46 biopsy specimens were HPV positive and 28 were HPV negative. They proposed that p16 protein expression in oral carcinogenesis may occur in relation to the functional inactivation of Rb protein by HPV infection. They also suggested that HPV pathogenesis by E6 and E7 oncoproteins deactivates Rb which thereby causes malfunction of p16. They concluded that there is a strong correlation between the presence of HPV and their overexpression in potentially malignant and malignant lesions of the oral mucosa[35].

Bouda M *et al* (2000) determined the role of HPV in oral carcinogenesis. They studied their presence in 53 potentially malignant and malignant oral lesions which includes 29 cases of hyperplasia, 5 cases of dysplasia, 19 cases of squamous cell carcinoma and 16 biopsies from healthy individuals using highly sensitive PCR, restriction fragment length polymorphism analysis, dot blotting and insitu hybridisation. They found that in all the different techniques used, at least one type of HPV which included HPV subtypes 16, 18, 33 and 58 were seen commonly. They concluded that there was an early involvement of HPV in oral carcinogenesis[36].

ORAL SUBMUCOUS FIBROSIS:

Pindborg *et al* (1966) proposed that OSF is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat. There are connective tissue changes present in OSF.

The early stage is characterized by a finely fibrillar collagen, dispersed with marked edema. The fibroblastic response is produced by fibroblasts which are plump of young cells with abundant cytoplasm. In later stages, the collagen present in connective tissue is moderately hyalinized, the amorphous change starting from the juxta-epithelial basement membrane. Occasionally, thickened collagen bundles are still seen separated by slight residual edema. The fibroblastic response is less marked and the fibroblasts are mostly having elongated spindle-shaped nuclei and scanty cytoplasm. Oral epithelium in the affected areas is markedly atrophic as compared with the thickness of normal oral epithelium. The rete pegs are completely lost. The buccal mucosa, normally non-keratinised are showing. The atrophy of the oral epithelium is probably secondary to the connective tissue changes[5].

PATHOGENESIS OF OSF:

Thavarajah R, *et al* in 2004 conducted a cytological study of copper in OSF. They found that high copper content present in areca nut plays a vital role in

pathogenesis of OSF. This study evaluates the copper-staining pattern of buccal epithelial cells in oral cytological smears of non-chewers, chewers and OSF. Copper appeared as shades of pale red within the cytoplasm of chewers and did not show any stain in non-chewers. Intense red stain was seen in OSF smears as dark granules within the cytoplasm. They concluded that intense staining of copper in OSF buccal smears in areca nut chewers support the role of copper in the pathogenesis of OSF[37].

Tilakaratne WM *et al* (2006) reviewed the etiology and pathogenesis of OSF from data taken from recent epidemiological studies. The studies provided evidence that arecanut is the main aetiological factor for OSF. A dose-dependent relationship was observed for both frequency and duration of chewing arecanut (without tobacco) in the development of OSF. Commercially freeze dried products such as pan masala, Gutkha and mawa have high concentrates of arecanut per chew and appear to cause OSF more rapidly than by self-prepared conventional betel quid that contain smaller amounts of arecanut. The authors hypothesized that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease. These chemicals appear to interfere with the molecular processes of deposition and/or degradation of extracellular matrix molecules such as collagen. In vitro studies on human fibroblasts using areca extracts or chemically purified arecoline support the theory of fibroblastic proliferation and increased collagen formation that is also demonstrable histologically in human OSF tissues[38].

MOLECULAR PATHOGENESIS OF OSF:

Initial events of the disease include chronic irritation of the oral mucosa due to constant betel quid chewing habit. This further leads to chronic inflammation. The normal physiologic events include activation of T cell and macrophages at the irritation site. Then there will be increase in cytokines IL6, TNF, IFα and increase in growth factor TGFβ. In normal physiologic process there will be constant production of collagen and degradation. In OSF there will be increase in collagen production and decrease in collagen degradation. In collagen production pathway TGFβ activates N procollagen peptides, procollagen precursor, procollagen genes. These in turn increase procollagen which further releases collagen in soluble and insoluble form. As a result there will be total increase in collagen production. In collagen degradation pathway TGFβ activates genes, tissue inhibitor of matrix metalloproteinase (TIMP) and plasminogen activator inhibitor (PAI). TIMP inhibits activated collagenase and PAI inhibits conversion of plasminogen into plasmin. Plasmin also inhibits conversion of procollagenase into collagenase as a result there will be decreased collagenase activity and collagen degradation. This leads to fibrosis of the oral mucosa and OSF results[39].

MALIGNANT TRANSFORMATION OF OSF:

Murti PR *et al* (1985) conducted a study in OSF affected individuals where 66 patients with OSF were followed-up for a period of 17yr (median observation 10 yr) in Ernakulam District, Kerala, India. Oral cancer developed in five (7.6%) patients. The malignant transformation rate in the same sample was 4.5% over a

15-yr observation period (median 8yrs). These findings impart a high degree of malignant potential of OSF[40].

Pillai R *et al* (1992) proposed that OSF is a condition with a high risk of malignant transformation. They suggested a multifactorial causation for the malignant transformation of OSF namely; genetic, carcinogenic, immunologic, viral, nutritional, and autoimmune possibilities, all of which also have been implicated in the development of oral cancer[41].

Jeng JH *et al* (2001) found that areca nut products induce mutagenic and genotoxic effects, in addition to inducing preneoplastic as well as neoplastic lesions in experimental animals. Areca nut should, thus, be highly suspected as a human carcinogen. The mutagenicity and genotoxicity of areca alkaloids has been detected by many short-term assays. It appears that areca nut toxicity is not completely due to its polyphenol, tannins and alkaloid content. Reactive oxygen species produced during auto-oxidation of areca nut polyphenols in the Betel Quid chewer's saliva, are crucial in the initiation and promotion of oral cancer. Nitrosation of areca alkaloids also produces areca nut-specific nitrosamines, that have been demonstrated to be mutagenic, genotoxic and are capable of inducing tumours in experimental animals[42].

Afroz N *et al* (2006) studied about the association between areca nut and malignant transformation of OSF. They concluded that arecoline in areca nut can induce fibroblast proliferation and collagen synthesis and may penetrate the oral mucosa to cause progressive cross linking of collagen. Tobacco chewing and smoking are not considered to play a role in the development of this disease[43].

Pundir S *et al* (2010) found the development of cancer from OSF in 2 cases. In patients with OSF, the oral epithelium becomes atrophic and thereby becomes more vulnerable to carcinogens. It is now accepted that chewing areca is the most important aetiological factor for developing OSF. The atrophic epithelium shows first an intercellular edema and later epithelial atypia associated with moderate epithelial hyperplasia. From then on, carcinoma may develop any time. It is suggested that OSF should be regarded as a condition that causes predisposition to the development of oral cancer[44].

p16 IN OSF:

Takeshima M *et al* (2008) studied the occurrence of hypermethylation in OSF. They found high frequency hypermethylation of p14, p15 and p16 in OSF and no hypermethylation in normal epithelium. No significant correlation was observed between p53 positive reactions and hypermethylation in any lesions. The hypermethylation was highly detectable even in p53 negative lesions, suggesting that hypermethylation of p14, p15 and p16 occur regardless of whether the lesions have p53 mutations or not. From all these findings they concluded that hypermethylation may be involved in the pathogenesis of OSF[45].

Fung L *et al* (2010) investigated the expression of Fragile Histidine Triad (FHIT) and p16 protein in 44 cases of OSF and 15 cases of OSF concomitant with OSCC and 8 cases of normal mucosa for evaluating the carcinogenesis in OSF. Results showed p16 expression in normal tissues, OSF and OSF concomitant with oral squamous cell carcinoma 100%, 72.7% and 40% respectively. From these results they proposed that loss of p16 is likely to be an early occurrence in the

carcinogenesis of OSF and the positive correlation between FHIT and p16 demonstrates that they may act together promoting the carcinogenesis of OSF[46].

ORAL SQUAMOUS CELL CARCINOMA:

Williams HK *et al* (2000) postulated that OSCC, the most common cancer of the oral cavity accounts for over 90% of malignant neoplasms. The incidence of oral cancer remains high and is associated with many deaths in both Western and Asian countries. Several risk factors for the development of oral cancer are now well known, including smoking, drinking and consumption of smokeless tobacco products. Genetic predisposition to oral cancer has been found in certain cases but its components are not yet entirely clear. In accordance with the multi-step theory of carcinogenesis, the natural history of oral cancer seems to be gradually evolving through transitional precursor lesions from normal epithelium to a full-blown metastatic phenotype. A number of genomic lesions accompany this transformation. Furthermore, several key genes have been implicated, especially well-known tumour suppressors like the cyclin-dependent kinase inhibitors, TP53 and RB1 protein and oncogenes like the cyclin family, Epidermal Growth Factor Receptor (EGFR) and Rouse Avian Sarcoma (RAS). Viral infections, particularly with oncogenic HPV subtypes and Epstein Barr Virus (EBV), can have a tumorigenic effect on oral epithelia[47].

Patel V *et al* (2001) reviewed the various diagnostic tools used in the detection of OSCC to understanding the molecular basis of cancer. This included head and neck squamous cell carcinoma-specific chromosomal alterations c(CAP), systematic identification of novel genes using head and neck cancer

genome anatomy project (HN-CGAP), characterisation of malignant, potentially malignant and normal cells by means of DNA microarray technology using laser capture mirodissection (LCM) which plays a vital role in detection of head and neck squamous cell carcinomas and their pathogenesis[48].

Perez OB *et al* (2006) summarised the alterations in genetic basis and disease progression of squamous dysplasia to cancer. They discussed the loss of heterozygosity (LOH) of 9p21 chromosomal region in the early stages of cancer. They discussed the genetic alterations involving in inactivation of tumour suppressor genes viz. p16INK4a, p53, cyclin D1, $p14^{ARF}$ and activation of proto-oncogenes viz FHIT, Rb, EGFR and RASSF1A respectively[49].

Massano J *et al* (2006) reviewed the relevant published data into 3 groups patient-, tumour-, treatment related factors. Tumour related factors included some less commonly studied factors viz. disease staging, extracapsular dissemination, resection margin free of disease and tumour thickness. Tumour molecular factors included several genes which includes tumour suppressor genes p16 and p53, overexpression of oncogenes PRAD1, EGFR and cyclins and cyclin dependent kinases involved in the cell cycle. They concluded that large scale study of the factors involved in prognosis of cancer must be done to evaluate the therapeutic strategies of cancer[50].

Scully C *et al* (2008) summarised the current diagnostic tools for detection of oral cancer which included biopsy and histopathological examination, vital staining, biomarkers, brush biopsy, DNA ploidy, optical systems and saliva-based

oral cancer diagnostics. They suggested certain other diagnostic tools like laser and light induced fluorescence spectroscopy, photo acoustic imaging, quantum dots, narrow band imaging, 2 photon fluorescence, tetrahertz imaging for the detection of oral cancer. They proposed that along with well-established imaging techniques other adjunct detection methods must be used to evaluate the early diagnosis and treatment of cancer[51].

Campo TJ *et al* (2008) summarised the importance of cytogenetic alterations which included LOH, microsatellite instability, epigenetic alterations like aberrant methylation of chromosomes involving cancer and precancer. They proposed that p16 methylation and mutation involves in 70% of head and neck squamous cell carcinomas. They also stated that the tumour-stroma interactions and the intercellular signalling pathways were involved in OSCC adding that signalling molecules, stromelysin, E-cadherin, oncogenes, tumour suppressor genes and viruses were responsible for tumorigenesis[52].

p16 IN ORAL SQUAMOUS CELL CARCINOMA:

Reed AL *et al* (1996) studied the expression of p16 in head and neck squamous cell carcinomas using immunohistochemistry and compared with microsatellite analysis, southern blot analysis and sequence analysis. They used 29 head and neck squamous cell carcinomas and the results showed loss of p16 expression in all 29 cases in immunohistochemistry. By other analysis, homozygous deletions and methylations were found in 24 cases of OSCC. From their results they concluded that there was higher frequency of p16inactivation in

squamous cell carcinomas and the inactivation may be due to homozygous deletions, point mutations and methylation of CpG islands[8].

Lazarus P *et al* (1998) studied the genetic mutations of p16 and p53 in oral squamous cell carcinomas and their association with specific genotypes using PCR. They found mutations in p53 exons 5-9 and p16 exons1-2 mutations. They found significant association of p53 with genotypes CYP1A1 and GSTM1 and no association of p16 with these genotypes. They concluded that mutations of these genes may be due to specific carcinogens and their association with specific genotypes which helps in the prognosis[53].

Sartor M *et al* (1999) studied the expression of cyclin D1, p16, Rb in OSCC cell lines using PCR. They did their study in 26 OSCC, nine premalignant lesions, three normal oral tissue samples and eight established OSCC cell lines for mutations in the p16/MTS1 gene. They found p16/MTS1 gene alterations in 5/26 (19%) primary tumours and 6/8 (75%) cell lines. They found that there is absence of p16/MTS1 expression in 18/26 (69%) OSCC, 7/9 (78%) premalignant lesions and 8/8 cell lines using western blot. Their data showed that p16/MTS1 mutations and loss of expression is common in oral cancer cell lines and less frequent in primary OSCC tumours. They found a different pattern of p16/MTS1 mutations in OSCC compared to other cancers with all the detected p16/MTS1 mutations resulting in premature termination codons or a frameshift. The RB protein expression in about half (44%) of OSCCs is inversely correlated with p16/MTS1 expression. They found genetic alterations which included LOH, CpG island aberrant methylation in most of the cancer cell lines[54].

Poi MJ *et al* (2001) studied the somatic mutations of INK4a-ARF locus in squamous cell carcinomas of head and neck which includes samples from pharynx, larynx, oral cavity and unclassified samples. They used PCR nonradioactive modification of single stranded conformational polymorphism (SSCP) termed cold SSCP in 100 squamous cell carcinomas. They found microdeletions, insertions, sequence alterations in 27 cases of squamous cell carcinoma in all the analysis. They found that many microdeletions and microinsertions are present along with the usual point mutations involved in p16 in cancer. They also proposed that these genetic alterations should be studied in a large scale to evaluate the importance of p16 in cancer. From the results they concluded that alterations in p16 and p14 resulted in complete mutation of INK4a-ARF locus which is prevalent in the squamous cell carcinoma of head and neck[55].

Yuen PW *et al* (2002) studied p16 expression in 225 squamous cell carcinoma using immunohistochemistry. They found decreased expression in 48% of the cases and more intense expression in tumours with aggressive nature. The expression of p16 gradually reduced as the staging of the tumour advanced. They proposed that p16 is not significantly related to sex, age but being an important factor in cell cycle its reduced expression in tumours with high rate of proliferation is obvious. They concluded that from their data p16 cannot be used as a prognostic factor and for detecting nodal metastasis and survival[56].

Geisler SA *et al* (2002) did a cohort study for the expression of p16 and p53 in 171 cases of squamous cell carcinomas under treatment. They found increased expression of p53 in majority of the cases. The cases expressing

alterations in both p16 and p53 were of no diagnostic importance. They concluded that p16 cannot be used as a prognostic indicator for survival and recurrence of cancer[57].

Viswanathan M *et al* (2003) studied promoter hypermethylation of p16, p15, hMLH1, MGMT and E-Cadherin in 51 oral squamous cell carcinomas using sensitive restriction-multiplex PCR method. They found aberrant methylation of atleast one gene in 75% of the cases and no methylation was observed in 25 oral squamous tissues. They proposed that the methylation may be due to somatic sequence alterations in the genes. They also emphasised the importance of methylation in cancers. The methylation in human cancer is distinct and need not be in the same region on subsequent formation. Particularly in p16, there is higher incidence of methylation in the promoter site of the gene which gives more information regarding the significance of p16. They concluded that detection of promoter methylation of any of these genes may help in the diagnosis of OSCC[58].

Ai L *et al* (2003) studied the p16 expression in 100 squamous cell carcinomas using immunohistochemistry, methylation specific PCR. They found 74% of cases showing p16 promoter methylation and 26% of cases showing loss of expression. They found that the epigenetic mechanism which causes methylation of p16 in OSCC is absent in case of lung cancer as found in earlier reports. Most of the cases from their study did not show any promoter methylation instead they revealed mutational changes which includes homogeneous deletions in common. From their results they concluded that p16 can be used as a predictive and prognostic factor for head and neck squamous cell carcinoma[59].

Nilsson K *et al* (2004) studied about the staining patterns and characterized the expression of p16INK4a, Rb-phosphorylation and proliferation in actinic keratosis, squamous cell carcinoma in situ and invasive squamous cell carcinoma based on the infiltrative behaviour. From their results they showed the expression of p16INK4a were weak and cytoplasmic p16INK4a staining and functional Rb in actinic keratosis, Strong nuclear and cytoplasmic staining in all carcinomas in situ in parallel with inactive Rb, invasive squamous carcinoma with a mixed p16 staining pattern where some tumours had strong cytoplasmic staining, large fraction of Rb-phosphorylated cells and high proliferation. They highlighted the upregulation of cytoplasmic p16INK4a in infiltrative cells compared to tumour cells. Similarly a strong and combined nuclear and cytoplasmic staining in infiltrative cells, was present in other invasive squamous cancers. They proposed that there is an independent potential behaviour of p16 to infiltrate or upregulate into the cytoplasm or nucleus which is also independent of tissue localisation or potential to affect proliferation[60].

Weinberger PM *et al* (2004) studied the expression of p16 in 123 oropharyngeal squamous cell cancer using immunohistochemistry. They found both overexpression of p16 in advanced stage and metastasising cancer cell lines and in local recurrences and loss of expression. They suggested that overexpression may be related to poor survival rate and the loss of expression may be related to prognosis and disease free survival. From their findings they proposed that p16 is a significant prognostic factor for and in local recurrences[61].

Lee JK *et al* (2004) analysed inactivation patterns of p16INK4a genes to evaluate the role of p16INK4a inactivation in the development of oral squamous cell carcinoma in 6 different cell lines. 3 kinds of inactivation pattern were examined (by homozygous deletion, promoter hypermethylation, point mutation). They used methylation specific PCR for their analysis. Their study concluded that inactivation patterns of p16INK4a were mainly homozygous deletion, promoter hypermethylation rather than point mutation in oral cancer cell lines. They also suggested that the therapeutic modalities of oral squamous cell carcinoma should be focussed on the type of inactivation[62].

Muirhead DM *et al* (2006) studied the p16 and retinoblastoma (Rb) gene expression in patient with 39 squamous cell carcinomas with resected margins using immunohistochemistry. They found retinoblastoma expression in 39 cases and p16 expression was seen in 6 cases. They found that there is no correlation between anatomic site and p16 changes instead they proposed that most of the oral premalignant lesion with reduced p16 expression is due to loss of heterozygosity. They proposed that reduced expression was seen in cases of nonkeratinised areas, poorly differentiated morphology and Rb gene expression in keratinised areas. They concluded that p16 and Rb expression were related to morphological findings and presence of keratinisation. They also proposed that changes in p16 also attribute to histologic grading and metastatic potential of the tumour cells[63].

Vairaktaris E *et al* (2007) studied the expression of p16 in oral squamous cell carcinoma, dysplastic and hyperplastic epithelium. They did their study in 37 animals which were taken as controls and 3 experimental groups in which they

induced carcinogenesis and sacrificed at 10, 14, 19 weeks. They tumour sections under histopathologic study showed decreased expression of p16 in hyperplasia, dysplasia and cancer in decreasing order. They proposed that increased amount of p16 protein during hyperplasia observed may be due to unscheduled transactivation of the p16 gene. Increased accumulation of p16 mRNA and protein is attributed to cellular senescence, oncogenic hyperactivity of RAS and inactivation of Rb. They concluded that p16 can be used as a prognostic indicator for cancer[64].

Dragomir LP *et al* (2012) studied the p53, p16 and Ki67 expression in 34 squamous cell carcinomas and dysplastic lesions using immunohistochemistry. Their results showed increased expression of Ki67 in all the cases, increased expression of p16 in dysplastic lesions and increased expression of p53 in tumour front of the dysplastic lesions. They suggested that p16 expression was mainly due to histopathologic prognostic factors. They concluded that these genes can be used as a marker for detecting the aggressive nature of the lesions[65].

HPV IN ORAL SQUAMOUS CELL CARCINOMA:

Hafkamp HC *et al* (2003) studied HPV association in premalignant lesions, head and neck squamous cell carcinomas, metastasising primary carcinomas using FISH, immunohistochemistry and SSCP analysis. They found HPV association and p16 expression in majority of the cases. They proposed that HPV association in head and neck tumours are attributed to the risk factor of tobacco and alcohol consumption. They also suggested that tonsils are usually prone to HPV, due to the easy accessibility of tonsil to HPV and its prompt

metaplastic capability as in uterine cervix, the presence of deep crypts and invaginations on its surface which facilitates the contact of HPV with the basal cells and lastly the immediate availability of cytokines from the underlying lymphocytes. They concluded that HPV plays an important role in head and neck tumorigenesis with or without use of tobacco and alcohol consumption[66].

Kreimer AR *et al* (2005) reviewed a series of cases to detect the HPV subtypes in the head and neck cancers. They did their study in 5,046 head and neck squamous cell carcinomas using PCR based detection methods and genotype determination. They found out that prevalence of HPV subtypes 16 and 18 was higher in oropharyngeal cancers and laryngeal cancers and in squamous cell carcinomas respectively in descending order. From the results they proposed that association of HPV in oropharyngeal cancers are much significant and needs further investigation for detailed prognostic factors[67].

Lewis JS *et al* (2010) studied the presence of HPV and p16 expression in squamous cell carcinomas using PCR and immunohistochemistry respectively. From their results they found the presence of HPV and p16 expression in most of the oropharyngeal squamous cell carcinomas. They proposed that there is a complete association of HPV in oropharyngeal cancers and p16 immunohistochemistry and that this can be used as the best diagnostic criteria for oropharyngeal cancers[68].

Joseph AW *et al* (2011) reviewed the shifting trends in the incidence and mortality of HPV associated head and neck squamous cell carcinoma (HNSCC).

They reviewed the complete epidemiology of HPV associated HNSCC. They proposed that there is an increase in HPV-HNSCC in the tonsils and/or base of tongue. They also emphasized that HPV-HNSCCs are completely unique from cancers which are associated with the risk factors like tobacco- and alcohol-related and shows a different tumour behaviour. They emphasized the importance of routine examination of HPV status in clinical setting which gives prognostic information and shows patients eligible for clinical trials for better treatment strategies[69].

O'Rorke MA *et al* (2012) conducted a metaanalysis of HPV association in head and neck cancer and their survival. They found reduction in mortality in HPV associated head and cancers compared to cancers not associated with HPV. They proposed that there was an increase in the association of HPV in head and neck cancers which gave way to detailed study in their molecular involvement[70].

Rautava J *et al* (2012) studied the prognostic significance of HPV associated head and neck cancers in 106 head and neck squamous cell carcinoma cases. HPV genotyping was done in all the cases. They also elucidated the presence of cofactors which also includes HPV subtypes 16, 18 and cytomegalovirus. They proposed that treatment outcomes of HPV associated head and neck cancers were comparatively poor. With the outcomes they also suggested that there is a strong association of HPV subtypes in head and neck cancers[71].

Dufour X *et al* (2012) reviewed the complete epidemiology, pathogenesis and biomarkers for HPV associated head and neck cancers. They emphasized the importance of different biomarkers in detecting the early involvement of HPV subtypes in head and neck cancers. The most significant biomarkers for HPV are p16, p53, EGFR and Cyclin D1[72].

Isayeva T *et al* (2012) reviewed the treatment outcomes in HPV positive and negative oropharyngeal cancer. They also presented the data on HPv16/18 transcriptional status in oral cavity carcinomas, salivary gland neoplasms using nested reverse transcription PCR. They detected HPV DNA in 4,195 oral cavity cancer patients, 1,712 laryngeal cancer patients, 120 sinonasal cancer patients, 154 nasopharyngeal cancer patients. Their data revealed significant association of HPV in orolaryngeal neoplasm. From the results they proposed that there is a strong viral interaction with orolaryngeal tumours which leads to progression of carcinoma[73].

HPV and p16:

Konig F *et al* (2007) studied the presence of HPV expression in 60 head and neck squamous cell carcinomas using tissue microarrays and their presence using insitu hybridisation, HPV positive expression using immunohistochemistry. their results showed HPV positivity in 10 carcinomas, HPV protein expression in 35 out of 60 carcinomas. From the results they showed statistically significant relationship between HPV and p16. Mutations of p16 has been found in cancers involving breast oesophagus, bladder tumours. From this study they have

proposed that HPV also may involve in the alterations in p16 gene which further leads to malignancy[74].

Kong CS *et al* (2009) studied the relationship between human papilloma viruses and head and neck squamous cell carcinomas to correlate the prognostic markers. They studied 82 squamous cell carcinomas by pyrosequencing analysis. They found that 44% of the tumours showed strong HPV positive signals. From their results they showed that there is a strong relationship between HPV and head and neck squamous cell carcinomas[75].

Chen ZW *et al* (2012) studied about the predictive findings of HPV based on equivocal p16 staining and percentage of positively stained cells. They did a retrospective study on cases which underwent p16 IHC and PCR. They studied a total of 392 cases which used p16 IHC and 26 cases which used p16 PCR. From these they studied 32 cases were studied for equivocal staining of p16 in IHC group and 15 cases in PCR group. Their results showed strong association of cytoplasmic and membrane staining with negative HPV status and faint, diffuse nuclear and cytoplasmic staining with positive HPV status. From their results they suggested that the staining pattern of p16 were commonly associated with HPV interaction[76].

RESULTS

SAMPLE CHARACTERISTICS:

10 controls of normal mucosa (Group I), 20 cases of OSF (Grousp II), 20 cases of leukoplakia (Group III) and 20 cases of oral squamous cell carcinoma (Group IV) were analysed for immunoreactivity of p16 protein.

Distribution of Age among Total Study Groups:

The cases were divided into six groups based on age. The groups were 21-30 years, 31-40 years, 41-50 years, 51-60 years, 61-70 years and above 70. In Group I out of 10 cases 6(60%) belonged to 21-30 yerars, 3(30%) belonged to 31-40 years and one (10%) belonged to 41-50 years. In Group II out of 20 cases 4(20%) belonged to 21-30 years, 9(45%) belonged to 31-40 years, 5(25%) belonged to 41-50 years, and 2 cases (10%) belonged to 51-60 years. In Group III out of 20 cases one (5%) belonged to 21-30 years, 5(25%) belonged to 31-40 years, 2(10%) belonged to 41-50 years, 7(35%) belonged to 51-60 years, 4(20%) belonged to 61-70 years and 1(5%) belonged to above 70 years. In Group IV out of 20 cases 4(20%) belonged to 31-40 years, 6(30%) belonged to 41-50 years, 4(20%) belonged to 51- 60 years, 3(15%) belonged to 61-70 years and 3(15%) belonged to above 70 years. There was a significant difference between the groups with respect to age ($p = 0.001$).(Table 1, Graph 1)

Distribution of Gender among Total Study Groups:

In Group I, 8(80%) were males and 2(20%) were females. In Group II, 19(95%) were males and one (5%) was female. In group III, 16 were males (80%) and 4(20%) were females. In Group IV, 18(90%) males and 2(10%) were females.

The was a significant difference between the groups with respect to gender (p = 0.455). (Table 2, Graph 2).

Distribution of Site among Total Study Groups:

In Group I, 7(70%) incisional biopsies were from buccal mucosa and 3(30%) were from gingiva. In Group II, all the 20(100%) cases were from buccal mucosa. In Group III, 15(75%) were from buccal mucosa, 4(20%) were from tongue and one (5%) was from lip commissures. In Group IV, 9(45%) were from the buccal mucosa, 6(30%) were from gingiva, one (5%) was from hard palate and 4(20%) were from tongue. There was a significant difference between the groups with respect to site (p = 0.022). (Table 3, Graph 3)

Distribution of Habits among Total Study Groups:

The habits in the study groups are categorised as chewing & drinking alone, drinking & smoking alone, chewing alone, chewing, drinking & smoking and no habits. Within those in Group I, among total 10(100%) of the case none of them had any habits. In Group II, 6(30%) had the habit of chewing & drinking alone, 9(45%) had the habit of chewing, drinking & smoking, 5(25%) had the habit of chewing alone. In Group III, 13(65%) had no habits, 5(25%) had the habit of drinking & smoking alone, one (5%) had the habit of chewing, drinking & smoking and one (5%) had the habit of chewing alone. In Group IV, 16(80%) had no habits, 3(15%) had the habit chewing, drinking & smoking, one (5%) had the habit of chewing alone. There was a significant difference between the groups with respect to habits (p = 0.001). (Table 4, Graph 4)

DISTRIBUTION OF p16 STAINING AMONG STUDY GROUPS:

Of the total number of cases subjected to p16 staining, 16(22.9%) cases showed positive staining and 54(77.1%) of the cases showed negative staining. In Group I, out of 10 cases 5(50%) showed positivity and 5(50%) showed negative staining. In Group II, out of 20 cases only 7(35%) were positive for p16 expression and 13(65%) of the cases showed negative staining. In Group III, out of 20 cases only one (5%) showed positivity and 19(95%) cases showed negative staining. In Group IV, out of 20 cases only 3(15%) showed positivity and 17(85%) of the cases showed negative staining. There was a significant difference between the groups with respect to p16 positivity ($p = 0.017$). (Table 5, Graph 5)

The following parameters were used to evaluate p16 staining in all the 4 groups:

- Staining intensity
- Staining pattern
- Percentage of cells stained
- Tissue localisation of the stain

Tissue Localisation of the Stain:

p16 staining was limited either to basal and suprabasal layers of the epithelium. Of the 70 cases examined, 6 showed positive staining in either basal or suprabasal layers or in both. In Group I, 4(40%) showed staining only in the basal layers of the epithelium and one (10%) showed staining in both basal and suprabasal layers of the epithelium. In Group II, 2(10%) showed staining only in the basal layers of the epithelium, 4(20%) showed staining only in the suprabasal layers of the epithelium, one (5%) showed staining in both basal and suprabasal layers of the epithelium. In Group III, one (5%) showed staining in the basal

layers of the epithelium. In Group IV 1(5%) showed staining in the basal layers of the epithelium and 2(10%) showed staining in the suprabasal layers of the epithelium. There was a significant difference between the groups with respect to staining pattern (p = 0.019). (Table 6, Graph 6)

Staining Intensity:

Each of the positively stained cases were graded as mild, moderate and intense based on the intensity of p16 immunostaining. Among the 4 groups, 9(12.9%) cases had mild intensity, 6(8.6%) cases had moderate intensity and 1(1.4%) case showed intense staining.

In Group I, 4(40%) showed mild intensity and 1(10%) showed moderate intensity of p16 stain. In group II, 3(15%) showed mild intensity, 4(20%) showed moderate intensity. In Group III, 1(5%) showed mild intensity. In Group IV, one (5%) showed mild intensity, one (5%) showed moderate intensity and one (5%) showed intense staining. There was a significant difference between the groups with respect to staining intensity (p = 0.040). (Table 7, Graph 7)

Staining Pattern:

p16 staining within each cell was localised to nucleus or cytoplasm or membrane or in combinations between them. Out of 16 cases, 4(5.7%) cases revealed cytoplasmic staining. 11(15.7%) revealed staining in both nucleus and cytoplasm and one (1.4%) showed staining in both cytoplasm and membrane.

In Group I, only one (10%) case showed staining in cytoplasm, 3(30%) cases showed staining in both nucleus and cytoplasm and one (10%) case showed

staining in both cytoplasm and membrane. In Group II, 2(10%) cases showed staining in cytoplasm and 5(25%) cases showed staining in both nucleus and cytoplasm. In Group III, only one (5%) case showed staining in nucleus and cytoplasm. In Group IV, one (5%) case showed staining in cytoplasm and 2(10%) cases showed staining in nucleus and cytoplasm. There was no significant difference between the groups with respect to staining pattern (p = 0.099). (Table 8, Graph 8)

Nuclear and Cytoplasmic Staining Grading:

Out of 5 cases in Group I 5 cases had negative cells per 100 cells examined and 2 cases had 1% to 25% of positive cells, 2 cases had 26% to 50% of positive cells and one case had 51% to 75% of positive cells. In group II out of 20 cases 13 cases had negative cells per 100 cells examined, 3 cases had 1% to 25% of positive cells and 4 cases had 26% yo 50% of positive cells. In Group III 19 cases had negative cells per 100 cells and one case had 1% to 26% of positive cells. In Group IV17 cases had negative cells per 100 cells examined, 2 cases had 1% to 25% of positive cells and one case had 76% to 100% of positive cells. (Table 9)

Staining Intensity Comparison between I And Group II:

Out of 5 cases in Group I, 4(40%) showed mild intensity and one (10%) showed moderate intensity. Out of 7 cases in Group II, 3(15%) showed mild intensity and 4(20%) showed moderate intensity. There was no significant difference between the groups with respect to staining intensity (p = 0.296). (Table 10)

Staining Intensity Comparison between Group II And Group IV:

Out of 7 cases in Group II, 3(15%) showed mild intensity and 4(20%) showed moderate intensity. Out of 3 cases in Group IV one (5%) showed mild intensity, one (5%) showed moderate intensity and one (5%) showed intense staining. There was no significant difference between the groups with respect to staining pattern ($p = 0.228$). (Table 11)

Staining Intensity Comparison between Group I And Group IV:

Out of 5 cases in Group I, 4(40%) showed mild intensity and one (10%) showed moderate intensity. Out of 3 cases in Group IV, one (5%) showed mild intensity, one (5%) showed moderate intensity and one (5%) showed intense staining. There was no significant difference between the groups with respect to staining pattern ($p = 0.080$). (Table 12)

Staining Pattern Comparison between Group I And Group II:

Out of 5 cases of Group I, one (10%) showed cytoplasmic staining, 3(30%) cases showed both nuclear and cytoplasmic stainingand one (10%) showed staining in cytoplasm and membrane. Out of 7 cases of Group II, 2(10%) showed cytoplasmic staining and 5(25%) staining in nucleus and cytoplasm. There was no significant difference between the groups with respect to staining pattern ($p = 0.510$). (Table 16)

Staining Pattern Comparison between Group II And Group IV:

Out of 7 cases of Group II, 2(10%) showed cytoplasmic staining and 5(25%) staining in nucleus and cytoplasm. In Group IV one (5%) showed staining

in cytoplasm, two (10%) showed nuclear and cytoplasmic staining. There was no significant difference between the groups with respect to staining pattern (p = 0.341). (Table 17)

Staining Pattern Comparison between Group I And Group IV:

Out of 5 cases of Group I, one (10%) showed cytoplasmic staining, 3(30%) cases showed both nuclear and cytoplasmic staining and one (10%) showed staining in cytoplasm and membrane. In Group IV one (5%) showed staining in cytoplasm, two(10%) showed nuclear and cytoplasmic staining. There was no significant difference between the groups with respect to staining pattern (p = 0.174). (Table 19)

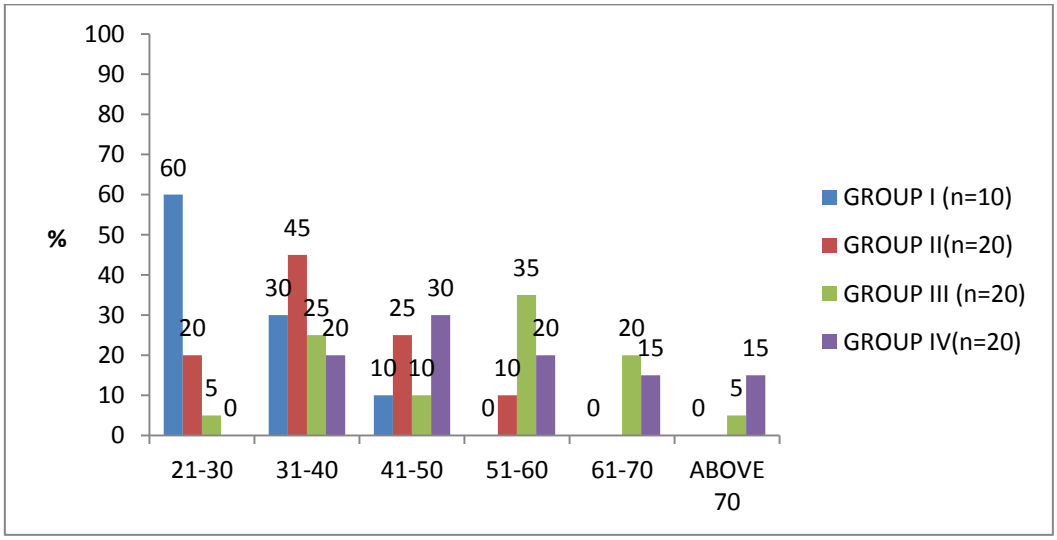
TABLES AND GRAPHS

TABLE 1: Age Distribution Among Total Study Groups

S.No	GROUPS	AGE						p VALUE
		21-30	31-40	41-50	51-60	61-70	ABOVE 70	
1.	GROUP I (n=10)	6(60%)	3(30%)	1(10%)	0	0	0	* 0.001
2.	GROUP II (n=20)	4(20%)	9(45%)	5(25%)	2(10%)	0	0	
3.	GROUP III (n=20)	1(5%)	5(25%)	2(10%)	7(35%)	4(20%)	1(5%)	
4.	GROUP IV (n=20)	0	4(20%)	6(30%)	4(20%)	3(15%)	3(15%)	

***p≤ 0.05 statistically significant**

GRAPH 1: Age Distribution Among Total Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

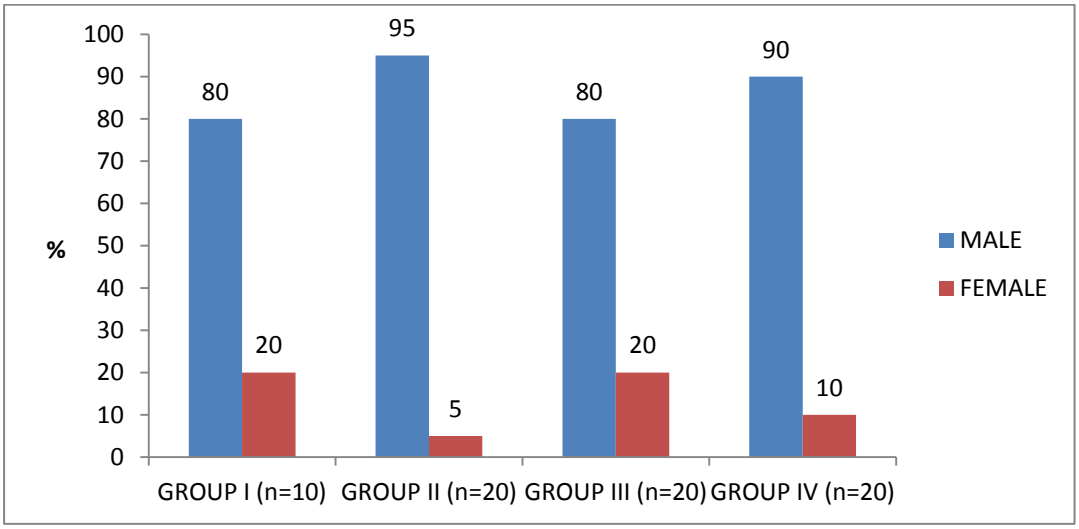
GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 2: Gender Distribution Among Total Study Groups

S.No	GROUPS	GENDER		p VALUE
		MALE	EMALE	
1.	GROUP I (n=10)	8(80%)	2(20%)	0.455
2.	GROUP II (n=20)	19(95%)	1(5%)	
3.	GROUP III (n=20)	16(80%)	4(20%)	
4.	GROUP IV (n=20)	18(90%)	2(10%)	

*p≤ 0.05 statistically significant

GRAPH 2: Gender Distribution Among Total Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

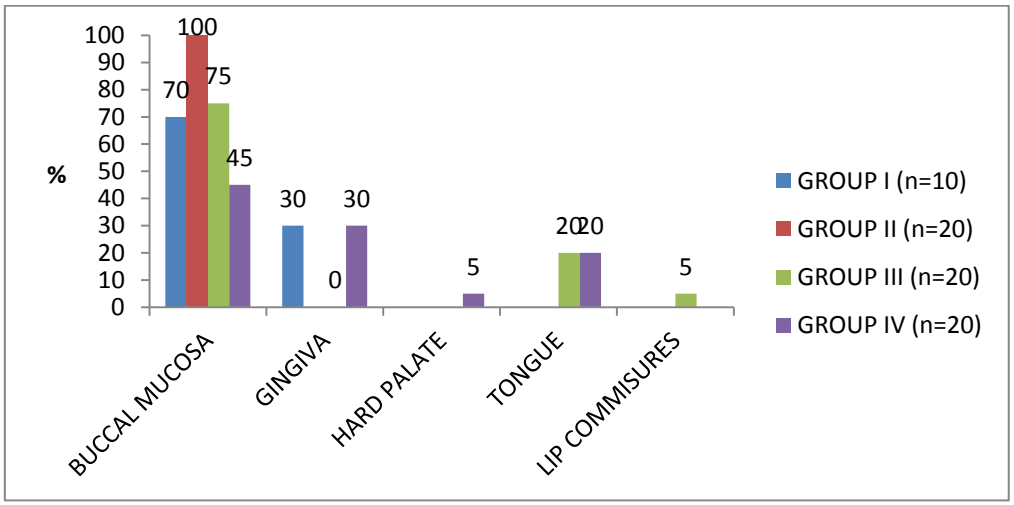
GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 3: Site Distribution Among Total Study Groups

S.No	GROUPS	SITE					p VALUE
		BUCCAL MUCOSA	GINGIVA	HARD PALATE	TONGUE	LIP COMMISURES	
1.	GROUP I (n=10)	7(70%)	3(30%)	0	0	0	*0.022
2.	GROUP II (n=20)	20(100%)	0	0	0	0	
3.	GROUP III ((n=20)	15(75%)	0	0	4(20%)	1(5%)	
4.	GROUP I (n=20)	9(45%)	6(30%)	1(5%)	4(20%)	0	

*p≤ 0.05 statistically significant

GRAPH 3: Site Distribution Among Total Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

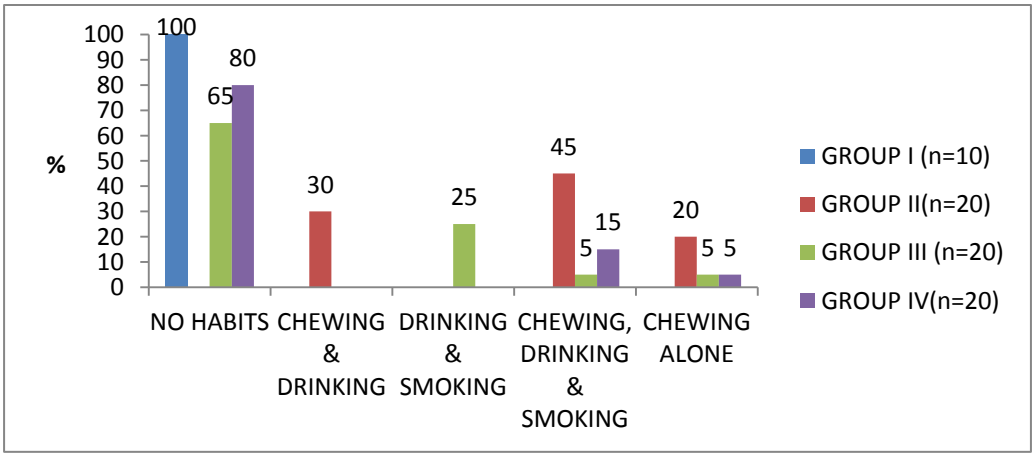
GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 4: Habit Distribution Among Total Study Groups

S.No	GROUPS	HABITS					p VALUE
		NO HABITS	CHEWING & DRINKING	DRINKING & SMOKING	CHEWING, DRINKING & SMOKING	CHEWING ALONE	
1.	GROUP I (n=10)	10(100%)	0	0	0	0	*0.000
2.	GROUP II (n=20)	0	6(30%)	0	9(45%)	5(25%)	
3.	GROUP III (n=20)	13(65%)	0	5(25%)	1(5%)	1(5%)	
4.	GROUP IV (n=20)	16(80%)	0	0	3(15%)	1(5%)	

*p≤ 0.05 statistically significant

GRAPH 4: Habit Distribution Among Total Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

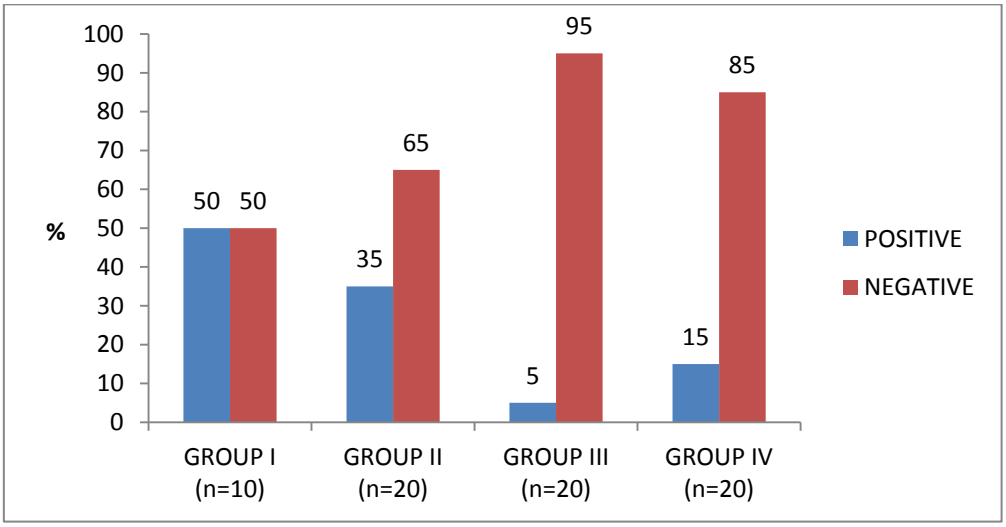
GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 5: Distribution Of p16 Staining Among Total Study Groups

S.No	DIAGNOSIS	p16 STAINING		p VALUE
		POSITIVE	NEGATIVE	
1.	GROUP I (n=10)	5(50%)	5(50%)	*0.017
2.	GROUP II (n=20)	7(35%)	13(65%)	
3.	GROUP III (n=20)	1(5%)	19(95%)	
4.	GROUP IV (n=20)	3(15%)	17(85%)	

*p≤ 0.05 statistically significant

GRAPH 5: Distribution Of p16 Staining Among Total Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 6: Tissue Localisation In p16 Positive Study Groups

S.No	GROUPS	TISSUE LOCALISATION				P VALUE
		BASAL	SUPRA BASAL	BOTH BASAL & SUPRABASAL	NEGATIVE	
1	GROUP I (n=10)	4(40%)	0	1(10%)	5(50%)	
2	GROUP II (n=20)	2(10%)	4(20%)	1(5%)	13(65.0%)	
3	GROUP III (n=20)	1(5%)	0	0	19(95.0%)	*0.019
4	GROUP IV (n=20)	1(5%)	2(10%)	0	17(85.0%)	

*p≤ 0.05 statistically significant

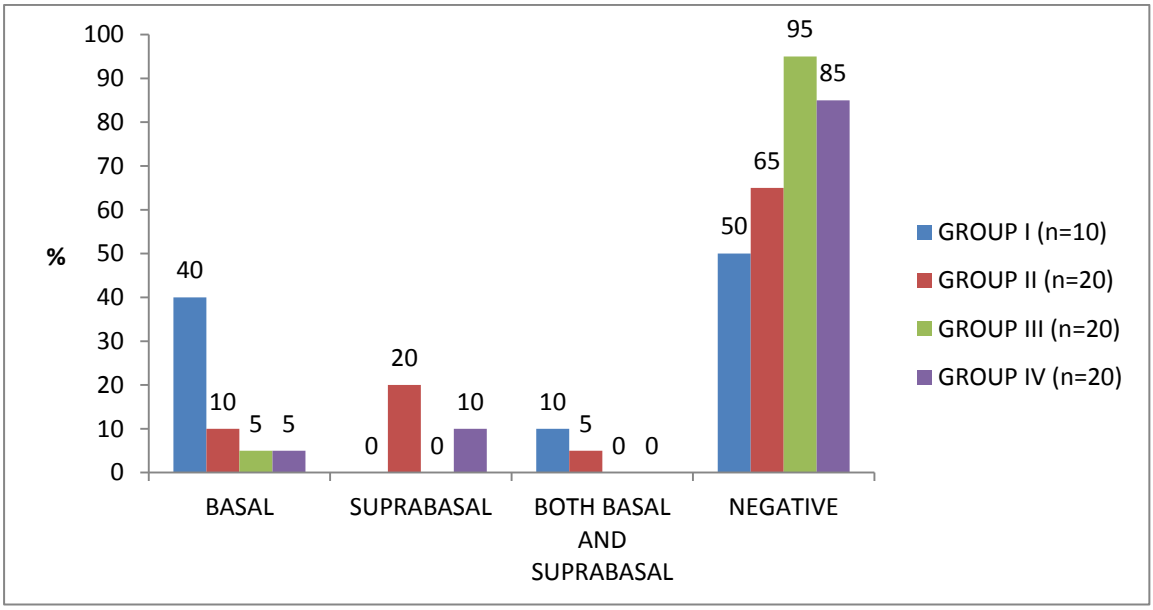
GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

GRAPH 6: Tissue Localisation In p16 Positive Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

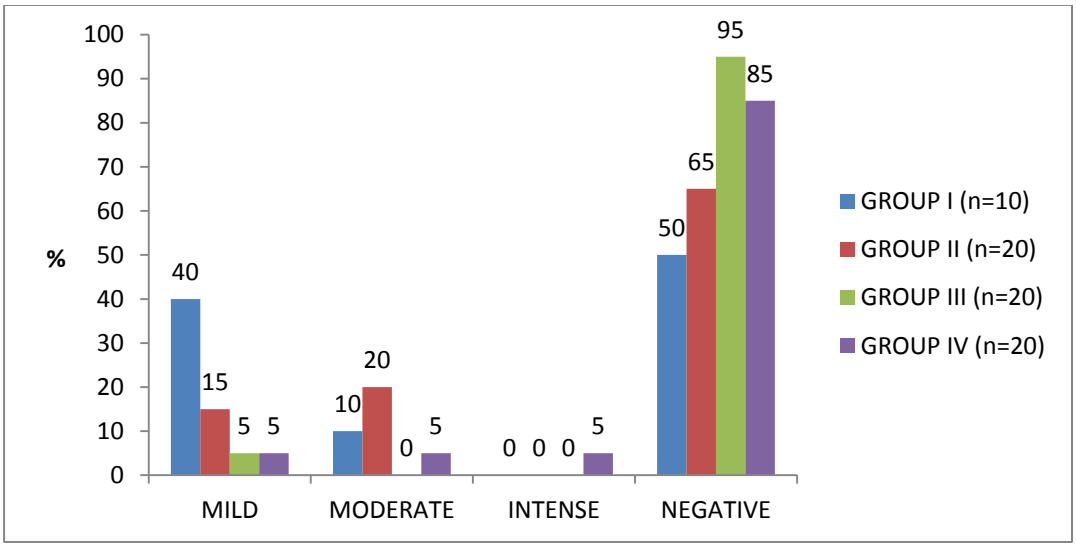
GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 7: p16 Staining Intensity Among p16 Positive Study Groups

S.No	GROUPS	p16 STAINING				P VALUE
		MILD	MODERATE	INTENSE	NEGATIVE	
1.	GROUP I (n=10)	4(40%)	1(10%)	0	5(50%)	
2.	GROUP II (n=20)	3(15%)	4(20%)	0	13(65%)	*0.040
3.	GROUP III (n=20)	1(5%)	0	0	19(95.0%)	
4.	GROUP IV (n=20)	1(5%)	1(5%)	1(5%)	17(85.0%)	

*p≤ 0.05 statistically significant

GRAPH 7: p16 Staining Intensity Among p16 Positive Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

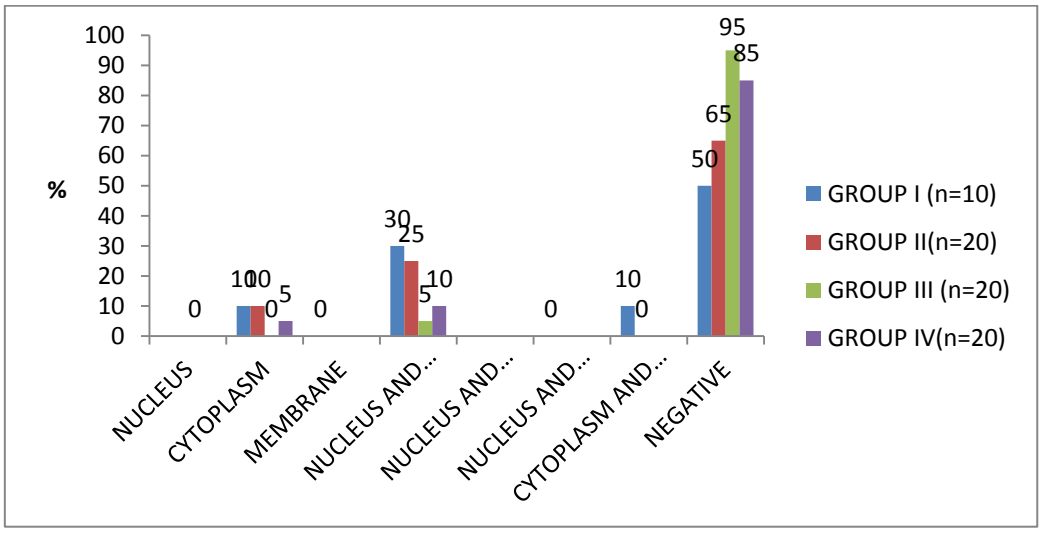
GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 8: Staining Pattern In p16 Positive Study Groups

S.No	GROUPS	STAINING PATTERN								p VALUE
		NUCLEUS	CYTO PLASM	MEMBRANE	NUCLEUS & CYTOPLASM	NUCLEUS & MEMBRANE	NUCLEUS, CYTOPLASM & MEMBRANE	CYTOPLASM & MEMBRANE	NEGATIVE	
1.	GROUP I (n=10)	0	1(10%)	0	3(30%)	0	0	1(10%)	5(50.0%)	0.099
2.	GROUP II (n=20)	0	2(10%)	0	5(25%)	0	0	0	13(65.0%)	
3.	GROUP III (n=20)	0	0	0	1(5%)	0	0	0	19(95.0%)	
4.	GROUP IV (n=20)	0	1(5%)	0	2(10%)	0	0	0	17(85.0%)	

*$p \leq 0.05$ statistically significant

GRAPH 8: Staining Pattern In p16 Positive Study Groups



GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 9: Nuclear And Cytoplasmic Staining Grading

GROUPS	**0**	**1+**	**2+**	**3+**	**4+**
GROUP I (n=5)	5	2	2	1	0
GROUP II(n=7)	13	3	4	0	0
GROUP III(n=1)	19	1	0	0	0
GROUP IV (n=3)	17	2	0	0	1

0 = negative

1+ = 1% to 25% of cells positive

2+ = 26% to 50%

3+ = 51% to 75%

4+ = 76% to 100%

TABLE 10: Comparison Of p16 Staining Percentage Positivity Among Positive Study Groups

S.No	GROUPS	PERCENTAGE POSITIVITY			P VALUE
		MEAN	S.D	MEDIAN	
1.	GROUP I (n=5)	53.33	5.25	55.97	0.925
2.	GROUP II (n=7)	51.80	3.43	52.92	
3.	GROUP III (n=1)	52.45	-	52.45	
4.	GROUP IV (n=3)	52.85	1.84	53.11	

*p≤ 0.05 statistically significant

GROUP I – NORMAL MUCOSA

GROUP II – OSF

GROUP III – EPITHELIAL DYSPLASIA

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 11: Staining Intensity Comparison Between Group I And Group II

GROUPS	STAINING INTENSITY		p VALUE
	MILD	MODERATE	
GROUP I	4(40%)	1(10%)	0.296
GROUP II	3(15%)	4(20%)	

*p≤ 0.05 statistically significant

GROUP I – NORMAL MUCOSA

GROUP II – OSF

TABLE 12: Staining Intensity Comparison Between Group II And Group IV

GROUPS	STAINING INTENSITY			p VALUE
	MILD	MODERATE	INTENSE	
GROUP II	3(15%)	4(20%)	0	0.228
GROUP IV	1(5%)	1(5%)	1(5%)	

*p≤ 0.05 statistically significant

GROUP II – OSF

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 13: Staining Intensity Comparison Between Group I And Group IV

GROUPS	STAINING INTENSITY			p VALUE
	MILD	MODERATE	INTENSE	
GROUP I	4(40%)	1(10%)	0	0.080
GROUP IV	1(5%)	1(5%)	1(5%)	

*p≤ 0.05 statistically significant

GROUP I – NORMAL MUCOSA

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 14: Staining Pattern Comparison Between Group I And Group II

S.No	GROUPS	STAINING PATTERN								p VALUE
		NUCLEUS	CYTO PLASM	MEMBRANE	NUCLEUS & CYTOPLASM	NUCLEUS & MEMBRANE	NUCLEUS, CYTOPLASM & MEMBRANE	CYTOPLASM & MEMBRANE	NEGATIVE	
1.	GROUP I (n=10)	0	1(10%)	0	3(30%)	0	0	1(10%)	5(50.0%)	0.51
2.	GROUP II (n=20)	0	2(10%)	0	5(25%)	0	0	0	13(65.0%)	

*p≤ 0.05 statistically significant

GROUP II – OSF

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 15: Staining Pattern Comparison Between Group II And Group IV

S.No	GROUPS	STAINING PATTERN								p VALUE
		NUCLEUS	CYTO PLASM	MEMBRANE	NUCLEUS & CYTOPLASM	NUCLEUS & MEMBRANE	NUCLEUS, CYTOPLASM & MEMBRANE	CYTOPLASM & MEMBRANE	NEGATIVE	
1.	GROUP II (n=20)	0	2(10%)	0	5(25%)	0	0	0	13(65.0%)	0.341
2.	GROUP IV (n=20)	0	1(5%)	0	2(10%)	0	0	0	17(85.0%)	

*p≤ 0.05 statistically significant

GROUP II – OSF

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

TABLE 16: Staining Pattern Comparison Between Group I And Group IV

S.No	GROUPS	STAINING PATTERN								p VALUE
		NUCLEUS	CYTO PLASM	MEMBRANE	NUCLEUS & CYTOPLASM	NUCLEUS & MEMBRANE	NUCLEUS, CYTOPLASM & MEMBRANE	CYTOPLASM & MEMBRANE	NEGATIVE	
1.	GROUP I (n=10)	0	1(10%)	0	3(30%)	0	0	1(10%)	5(50.0%)	0.174
2.	GROUP IV (n=20)	0	1(5%)	0	2(10%)	0	0	0	17(85.0%)	

*p≤ 0.05 statistically significant

GROUP II – OSF

GROUP IV – ORAL SQUAMOUS CELL CARCINOMA

PHOTOGRAPHS

FIGURE: 1 ANTIBODY KIT

FIGURE: 2 ARMAMENTARIUM

FIGURE 3 : Positive control: Squamous Cell Carcinoma – cervix (H&E - x4)

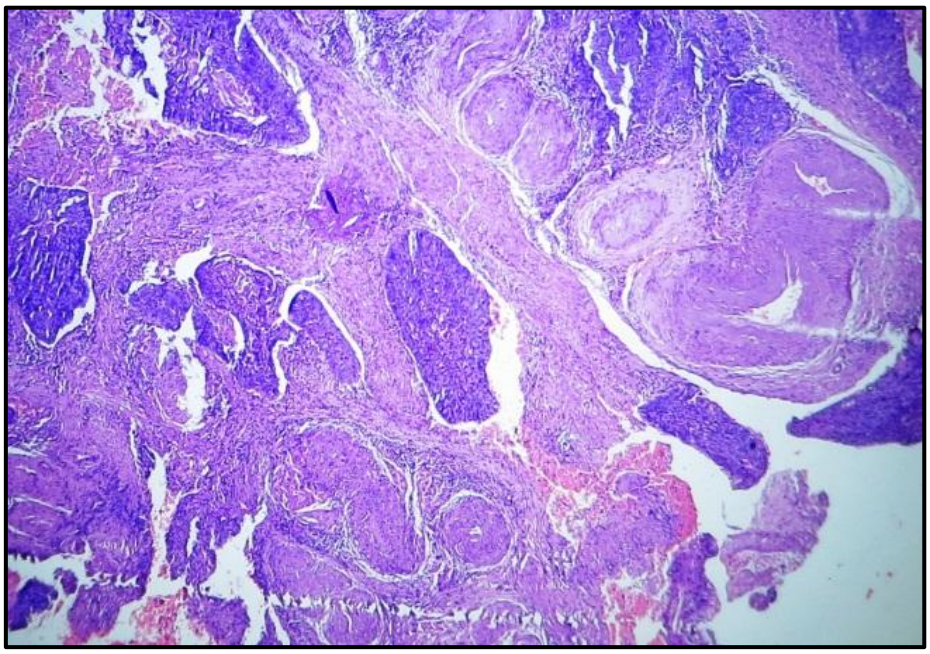


FIGURE 4 : Positive control: Squamous Cell Carcinoma – cervix (H&E - x10)

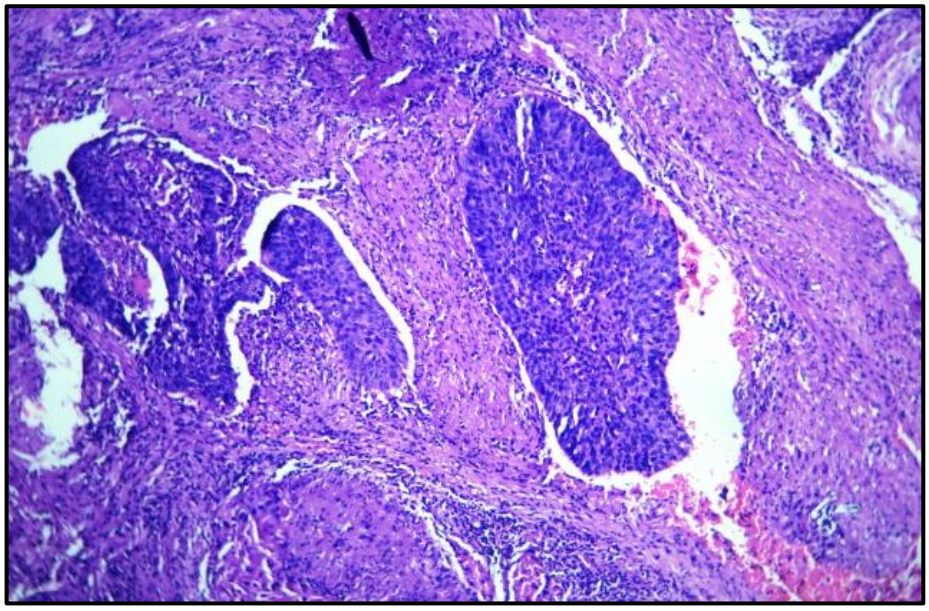


FIGURE 5 : Positive control: Squamous Cell Carcinoma – cervix (H&E - x40)

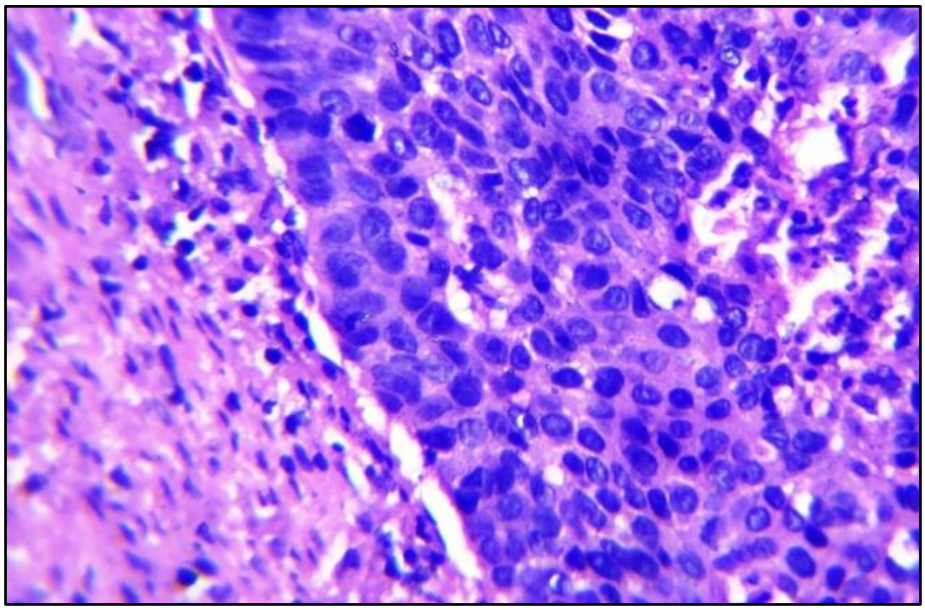


FIGURE 6 : Positive control: Squamous Cell Carcinoma – cervix (p16 stain - x4)

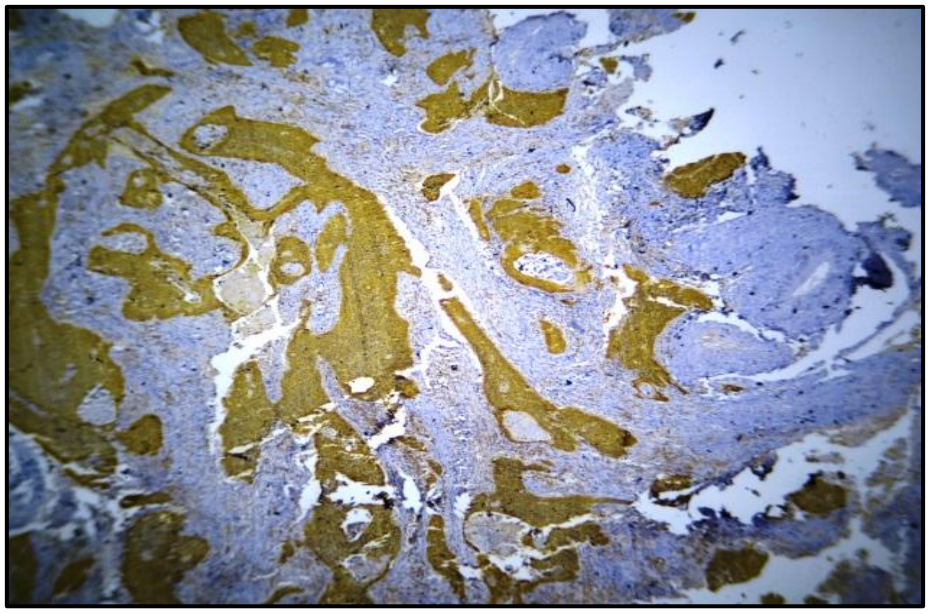


FIGURE 7 : Positive control: Squamous Cell Carcinoma – cervix (p16 stain - x10)

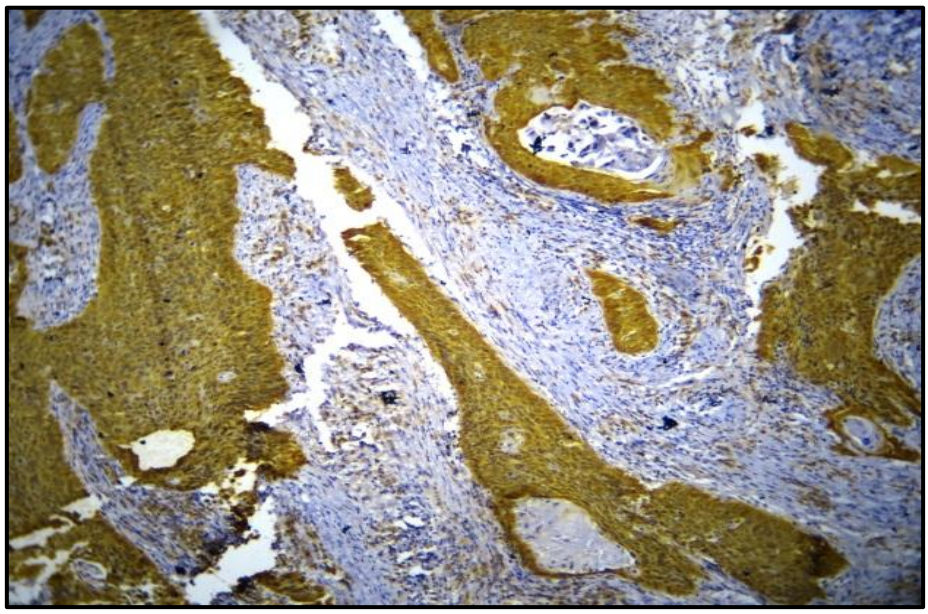
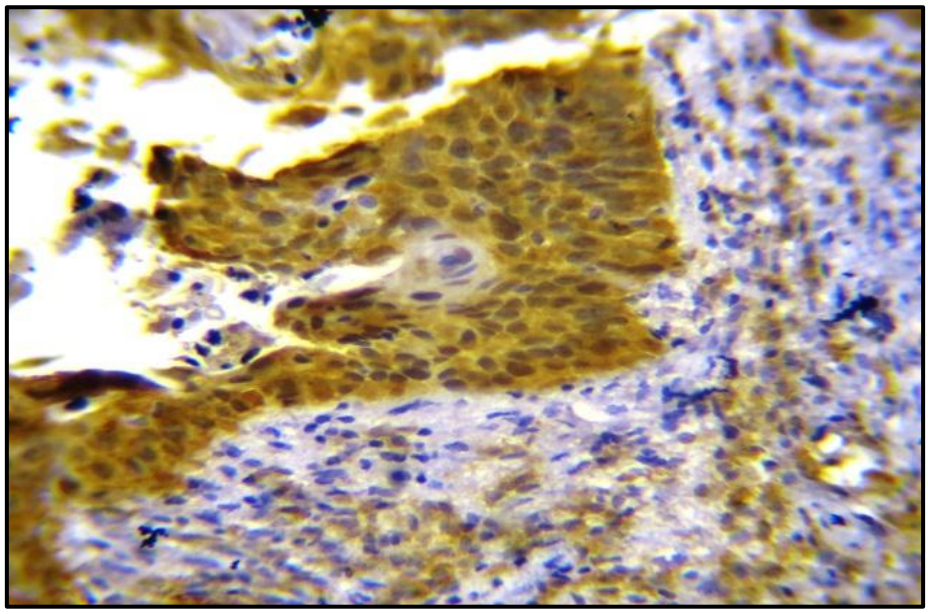
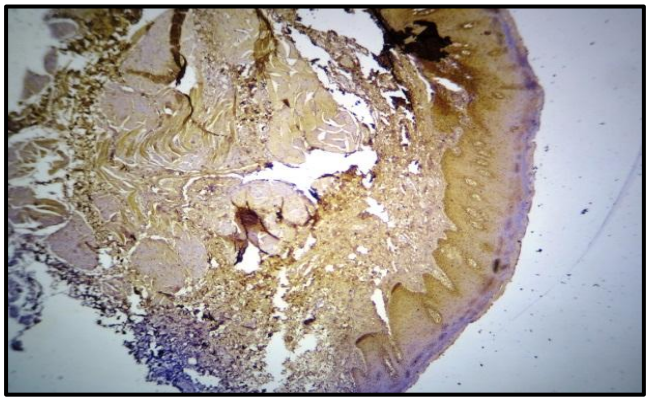


FIGURE 8 : Positive control: Squamous Cell Carcinoma – cervix (p16 stain - x40)



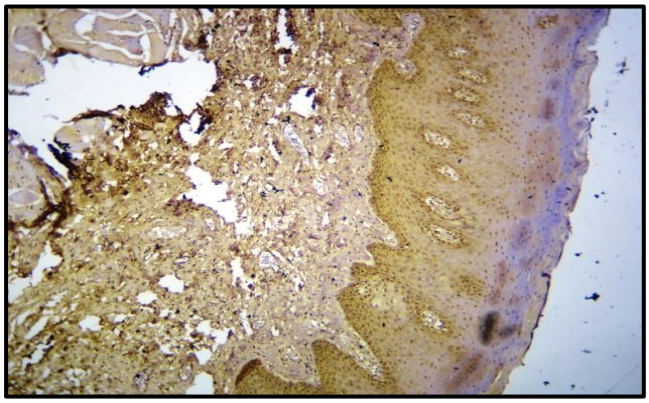
Nuclear and cytoplasmic staining

FIGURE 9 : p16 staining in the basal and suprabasal layers of epithelium in normal oral mucosa (Group I - x4)



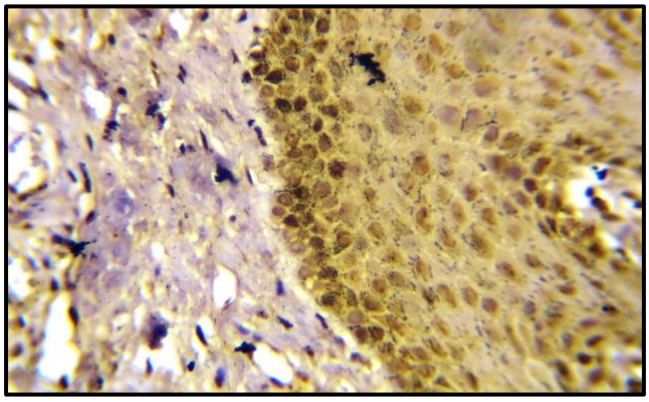
Nuclear and cytoplasmic staining

FIGURE 10 : p16 staining in the basal and suprabasal layers of epithelium in normal oral mucosa (Group I - x10)



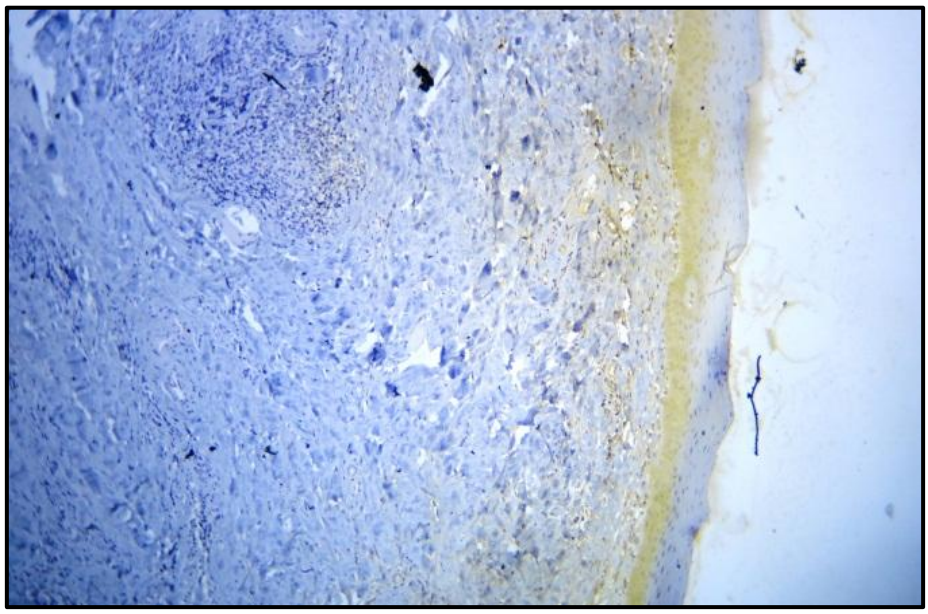
Nuclear and cytoplasmic staining

FIGURE 11 : p16 staining in the basal and suprabasal layers of epithelium in normal oral mucosa (Group I - x40)



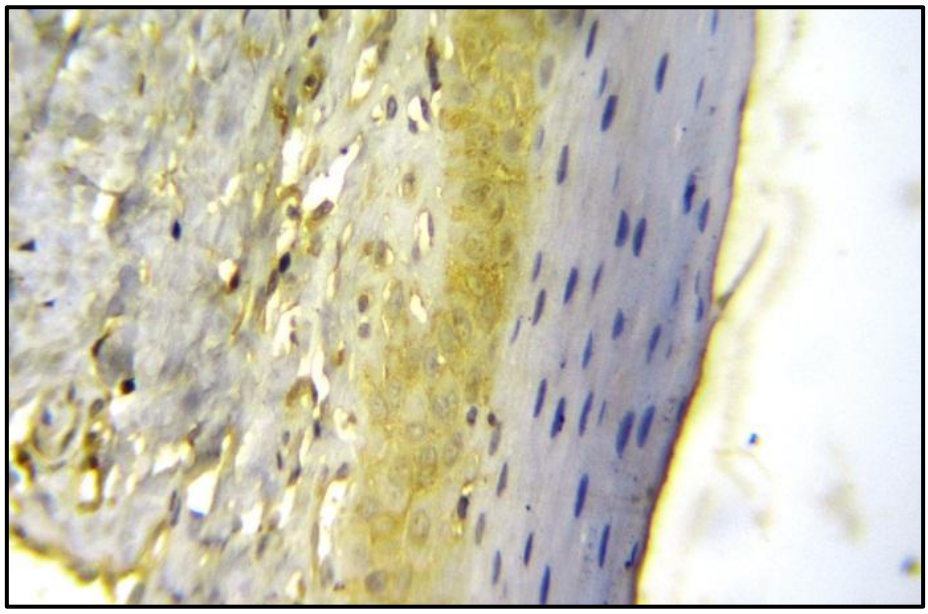
Nuclear and cytoplasmic staining

FIGURE 13 : p16 staining in the basal layers of epithelium in OSF (Group II - x10)



Cytoplasmic staining

FIGURE 14 : p16 staining in the basal layers of epithelium in OSF (Group II - x40)

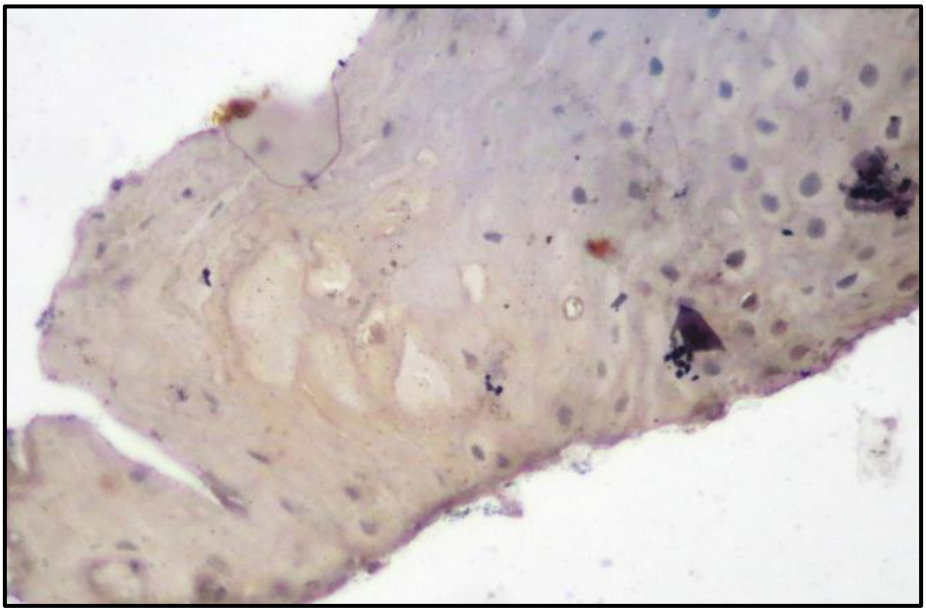


Cytoplasmic staining

FIGURE 16 : p16 staining in the basal layers of epithelial dysplasia (Group III - x10)

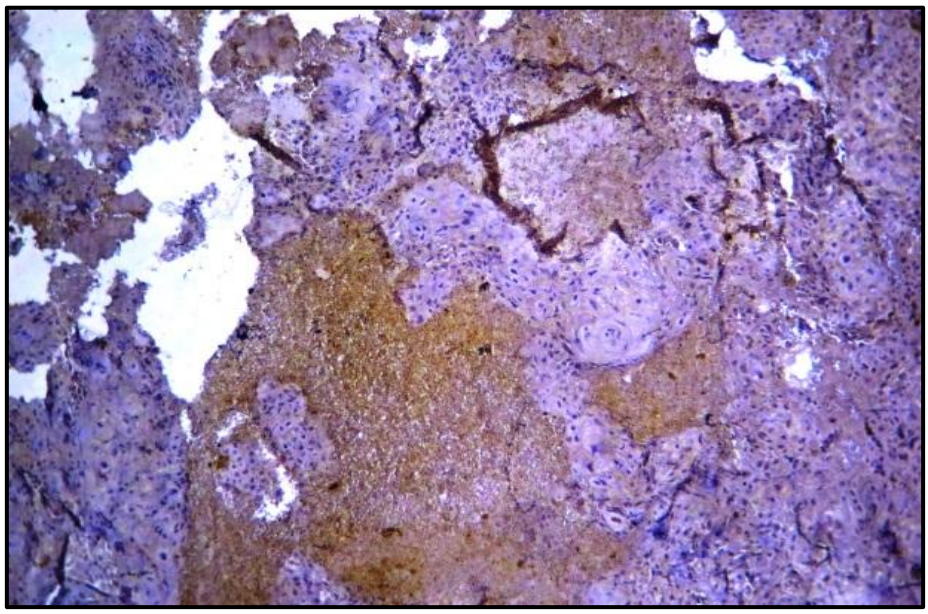
Cytoplasmic staining

FIGURE 17 : p16 staining in the basal layers of epithelial dysplasia (Group III - x40)



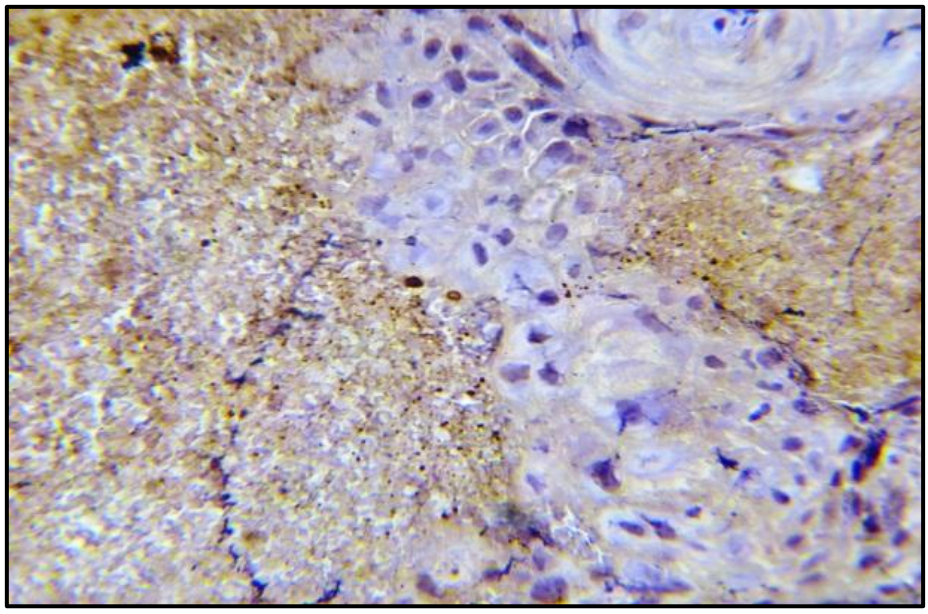
Cytoplasmic staining

FIGURE 18 : p16 staining in OSCC (Group IV - x10)



Nuclear and cytoplasmic staining

FIGURE 19 : p16 staining in OSCC (Group IV - x40)



Nuclear and cytoplasmic staining

DISCUSSION

Oral squamous cell carcinoma (OSCC), is the sixth most common cancer worldwide and the third most common form of cancer in the developing countries. The incidence of OSCC has increased due to increased risk factors such as tobacco and alcohol. OSCC usually arise from cells undergoing genetic and epigenetic alterations particularly in oncogenes or tumour suppressor genes[76].

The gene methylation is a common epigenetic event in the mammalian genome which involves addition of methyl group to the cytosol ring of the DNA base pairs resulting in CpG dinucleotide. Gene methylation also forms small unmethylated regions called CpG islands. There is increasing evidence suggesting the involvement of hypermethylation of CpG islands in different neoplasms. Hypermethylation denotes repeated methylation of CpG islands in the promoter site region of the p16 gene[10,11].

Carcinogenesis is a process involving dysregulation of cell cycle. p16 cyclin dependent kinase inhibitor (CKI) inhibits cyclin dependent kinases 4,6 and its complex with cyclin D. Thereby it also inhibits dephosphorylation and suppression of retinoblastoma (Rb) protein and also control other transcription factor responsible for transcription. All these activities together are known as p16INK4a/CDK4/cycD1/Rb pathway[66].

p16 immunohistochemistry has been shown to be a reliable marker for squamous dysplasia in uterine cervix[66,67]. In uterine cervix, p16 is overexpressed

due to infection of oncogenic strains of HPV. p16 overexpression is due to inactivation of pRb by HPV E7 oncoprotein. pRb-E7 complex releases E2F transcription factor which inhibits p16 gene transcription and translation resulting in increased expression. It has been shown that it can be also used for diagnosing dysplasia in cervix.

In oral cavity also, similar overexpression of p16 due to inactive Rb by HPV leading to malignant transformation has been reported[63].

It has been reported recently that overexpression of p16 in oral potentially malignant and malignant lesions has also been attributed to gene inactivating mechanisms such as hypermethylation, homozygous deletion and point mutation[10,16,74].

Given the fact that p16 has a vital role in the carcinogenesis, its association with HPV and its involvement in OSCC of head and neck it is important to identify the expression of p16 in oral potentially malignant and malignant lesions[39].

Given the known association of p16 and HPV in the dysplastic lesions; histopathologically those cases which were suggestive of HPV were included in the study.

SAMPLE CHARACTERISTICS:

There was a significant difference in the age of the patients enrolled between the 4 groups wherein 65% of patients in OSF were in the age group of

21-40 years, 35% of patients with epithelial dysplasia and 50% of patients with OSCC belonged to age group of 31-50 years. These differences in age group in OSF and epithelial dysplasia and OSCC could be due to the fact that, those who were chewing arecanut and subsequently developed OSF belonged to the younger age group[10].

There was a significant difference in gender among the groups and we would like to highlight that in group II, 95% were males compared to females, this is because of the prevalence of chewing arecanut was more commonly observed in males than females.

There was a significant difference in the site distribution among the study groups. Though majority of the cases were from buccal mucosa 25% in group III and IV were from the tongue.

Prevalence of habit varies significantly between the groups. The most interesting observation was that, of the randomly selected cases 65% in group III and 80% in group IV were without any habits.

p16 EXPRESSION IN EPITHELIAL DYSPLASIA:

Bradley *et al* stated that frequent inactivation of p16 during early carcinogenesis has necessitated the further investigations towards utilising p16 as a surrogate marker for dysplasia[32]. Immunohistochemical evaluation of oral premalignant and malignant lesions for p16 expression has given variable results

with some studies showing reduced expression[7,8] and others showing increased expression[62].

In our study, only 1 case of epithelial dysplasia expressed p16 and all others did not. Five cases of normal mucosa expressed p16 and 5 did not. The repeated loss of p16 gene expression in dysplastic mucosa could be expected to result in reduction of p16 protein expression in dysplasia when compared to normal mucosa.

In our study, the staining was limited to basal and suprabasal layer. This finding was similar to that of Bradley *et al* who reported that when the staining of p16 was present, it was confined to the basal and suprabasal cell layers in both normal and markedly dysplastic mucosa with no cases showing full thickness positivity. These findings indicate heterogenous expression of p16 within morphologically homogenous tissue. And thus, we agree with Bradley *et al* that p16 cannot be reliable in differentiating between normal and dysplastic mucosa[32].

It has been established that in uterine cervix, squamous dysplastic proliferative lesions frequently over express p16 with infection of high risk HPV types wherein p16 over expression has been attributed to Rb-E2F complex which normally inhibits transcription of p16 gene. Expression of HPV E7 results in excessive and dysregulated transcription and translation of p16.

It is now established that overexpression of p16 may serve as a biomarker for HPV induced oral dysplasia and carcinoma[36,74].

Mingli *et al* gave an alternative explanation to the reduced expression of p16 stating that epigenetic mechanisms such as aberrant methylation of p16, DAP-K and MGMT genes could play a role in progression of premalignant lesions to cancer[34].

Takeshima *et al* reported hypermethylation in 18% of mild dysplasia cases and 55% of severe dysplasia cases. Their study also concluded that hyper methylation may be involved in the pathogenesis of epithelial dysplasia[45].

p16 EXPRESSION IN OSF:

Few studies have explained the role of p16 in the pathogenesis of OSF. In our study, expression of p16 was seen in 35% of cases of OSF. None of the OSF cases exhibited epithelial dysplasia. Takeshima *et al* studied 10 cases of OSF without epithelial dysplasia and they assessed CpG island hypermethylation by methylation specific PCR method.70% of their cases expressed CpG islands by hypermethylation. This hypermethylation may lead to the suppression of transcription which induces malignant transformation[45]. The detection of hypermethylation in OSF may predict a risk of malignant transformation.

Wing YP *et al* explored and stated that the HPV status of tumours not staining strongly for p16 is difficult to interpret and may require a molecular technique. They further attempted to determine the staining pattern in equivocal p16 staining and its correlation to the percentage of positively stained tumour cells and analyse if it is predictive of HPV status. Their study showed strong association of membranous and cytoplasmic staining of isolated cells with

negative HPV status and concomitant faint diffuse nuclear, cytoplasmic staining with positive HPV status. HPV negative cases had only 30% positively stained tumour cells. HPV positive cases had 50%-90% positively stained tumour cells. They concluded that a diffuse nuclear and cytoplasmic staining pattern regardless of intensity is associated with HPV positivity. The HPV positive cases determined by staining pattern were also associated with higher percentage of tumour cells. When we extrapolate the results from Wing YP *et al* to our study of OSF, of the 7 cases which expressed p16 five cases showed nuclear and cytoplasmic expression and 2 showed only cytoplasmic expression[56]. A diffuse concomitant cytoplasmic and nuclear staining with 50% of cells showing p16 positivity could probably be due to coexisting HPV infection as stated by Chen ZW *et al*[76].

Muirhead et al in their study which they had done to correlate the expression of p16 and retinoblastoma protein stated that there could be a possible link between morphology and cell cycle regulation protein expression in squamous cell carcinoma of oral cavity. They stated that correlation of p16 to a morphological finding was unexpected. Their study showed that of the 6 tumours which were positive for p16 expression (or) negative for RB protein expression showed a marked non keratinising appearance or poor differentiation. Thus the tumours that exhibited p16 had significant characteristics of cells with predominantly non keratinised poorly differentiated morphology[63]. In our study there were significant differences between the 4 groups with respect to localised staining in basal and suprabasal layers. Of the 7 cases of OSF, four of them showed suprabasal staining. Based on the findings of Muirhead *et al* we could consider these cases could represent non keratinising appearance and this could be

indicative of poor differentiation. Thus we postulate that p16 expression in OSF could be an indicator of malignant transformation[63].

We postulate that p16 could probably be involved in the malignant transformation of OSF to OSCC. Alternatively, we should also elicit the role of HPV in OSF, given the p16-HPV pathway which has been strongly proven in cervical cancers[48].

p16 EXPRESSION IN OSCC:

Only15% of OSCC in our study expressed p16.WingYP*et al* in their study of HNSCC reported 48% of reduction in p16 expression. Based on this finding, they concluded that p16 expression is frequently reduced in HNSCC[56]. Fregonesi *et al* also correlated the expression of HPV with p16 and concluded that high risk HPV types are involved in p16 overexpression due to viral integration and malfunction of tumor suppressor protein contributing to multistep oral carcinogenesis[35].

Nilsson *et al* characterised the expression of p16, Rb phosphorylation and proliferation in invasive SCC of skin. The expression of p16 varied between the lesions. Invasive SCC showed nuclear and cytoplamic p16 expression pattern where some tumours had a strong cytoplasmic p16 expression. This staining was confined by Western Blot and band corresponding to SCC exhibiting only cytoplasmic p16 staining by IHC which indicated a true p16 staining in the cytoplasm. They could not elicit a reason as to why p16 was localised to

cytoplasm but proposed that it could be caused by mutation prohibiting translocation to the nucleus[60].

In our study of the 3 OSCC cases which expressed p16, two cases had both nuclear and cytoplasmic staining. Based on the results of Wing YP *et al* we interpret that one case of OSCC which had both nuclear and cytoplasmic staining also had more than 76% of cells expressing p16 and this finding could be probably associated with HPV infection.

Nilsson *et al* contributed to explain that the exclusive cytoplasmic staining of p16 could be due to its mutation prohibiting the translocation to the nucleus. They also stated that nuclear and cytoplasmic staining is due to combination of high proliferation, lack of Rb-phosphorylation and high p16INK4a expression therefore indicating the presence of an inactive Rb-pathway[60].

Lewis *et al* in a study of 239 cases of oropharyngeal OSCC to assess risk stratification found that 78% of cases were positive for p16. Of these 74% were positive for HPV by ISH. They stated that overexpression of p16 occurs as a result of degradation of Rb by HPV E7 oncoprotein, as Rb normally suppress p16 transcription and lack of Rb leads to marked overexpression of p16. In addition p16 is a tumour suppressor protein with a normal function of inhibiting cyclin dependent kinases. It is absent or weakly expressed in most non HPV related head and neck squamous cell carcinoma because the gene is mutated, deleted or methylated. They also stated that p16 is not the optimal biomarker for oropharyngeal squamous cell carcinoma (OPSCC) because it lacks the specificity

for HPV as p16 can be overexpressed by other mechanisms also. There are certain minority of OPSCC which are strongly p16 positive but are HPV negative. They further added that it could still be conceivable that p16 in their HPV negative neoplasms could have been involved in carcinogenic process of these tumours. They speculated that HPV might have been shed by the tumour cells. They stated that the tumour could develop independently from HPV and that they have innate p16 overexpression[68]. Based on their data they proposed that p16 positive, HPV negative oropharyngeal squamous cell carcinomas had better survival than p16 negative squamous cell carcinomas.

Observational findings in our study showed that in addition to the positively stained normal mucosa, the diffuse nuclear and cytoplasmic staining pattern in OSF and OSCC were indicative of a possible HPV association of these lesions. The absence of p16 expression in dysplastic lesions should be further evaluated in considering p16 as a marker to differentiate dysplastic mucosa from normal mucosa.

SUMMARY AND CONCLUSION

- A total of 70 patients were included in this study, comprising of 10 cases of normal oral mucosa (group I), 20 cases of OSF (group II), 20 cases of leukoplakia (group III) and 20 cases of oral squamous cell carcinoma (group IV).
- In group I, 70% of biopsies were taken from the buccal mucosa, 30% were from the gingiva.
- In group II, all the biopsies were taken from the buccal mucosa.
- In group III 65% of biopsies were taken from the buccal mucosa, 20% were from tongue and 10% from gingiva and 5% from lip commissures.
- In group IV 45% biopsies were taken from buccal mucosa, 30% were from gingiva, 20% were from tongue and 5% from hard palate.
- In group II 45% of patients had chewing tobacco, drinking and smoking habits and 30% of patients had the habit of chewing tobacco and drinking and 25% of patients had the habit of chewing tobacco alone.
- In group III 65% of patients had no oral habits, 25% of patients had the habit of drinking and smoking, 5% of patients had the habit of chewing tobacco, drinking and smoking and 5% of patients had the habit of chewing alone.
- In group IV, 80% of patients had no oral habits, 15% of patients had the habit of chewing tobacco, drinking and smoking and 5% of patients had the habit of chewing alone.
- In group III all the 20 cases showed features of dysplasia histologically, with 80% mild dysplasia, 15% moderate dysplasia and 20% severe dysplasia.

- Out of 20 cases in group IV, 55% were well differentiated OSCC, 30% were moderately differentiated OSCC, 15% were poorly differentiated OSCC.
- All the 70 samples were examined for the expression of p16 monoclonal antibody immunohistochemically using Poly Horse Radish Peroxidase.
- p16 staining was evaluated by counting the percentage of cells stained per 100 cells examined, by recording the intensity of the stain and also by examining the cellular location and nature of the stain.
- 35% in group II, 5% in group III, 15% in group IV showed positivity when compared to 50% of controls in group I.
- Out of 70 cases 12.9% of cases showed mild intensity, 8.6% of cases showed moderate intensity and 1.4% of cases showed intense staining intensity.
- Tissue localisation of the stain in the basal and suprabasal layers showed significant difference between the groups with four cases of OSF exhibiting suprabasal staining. Suprabasal staining of p16 could be indicative of loss of keratinisation and poor differentiation and thus could be an early marker of malignant transformation of OSF.
- The pattern of p16 staining(70 cases) showed 4(5.75%) cases having cytoplasmic staining, 11(15.7%) cases having nuclear and cytoplasmic staining and one(1.4%) case had cytoplasmic and membrane staining.

CONCLUSION:

In this study we addressed the association particularly between p16 and OSF, epithelial dysplasia and OSCC. The results of this study with respect to OSF data, highlights that p16 could play a role in malignant transformation of OSF and we hypothesize that it could be associated with HPV.

Further studies should ascertain the HPV status of the cases to be included, with a larger sample size to establish and understand if p16 expression could have a role in oral potentially malignant lesions and OSCC.

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ANNEXURE - I

From,

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi,

Chennai

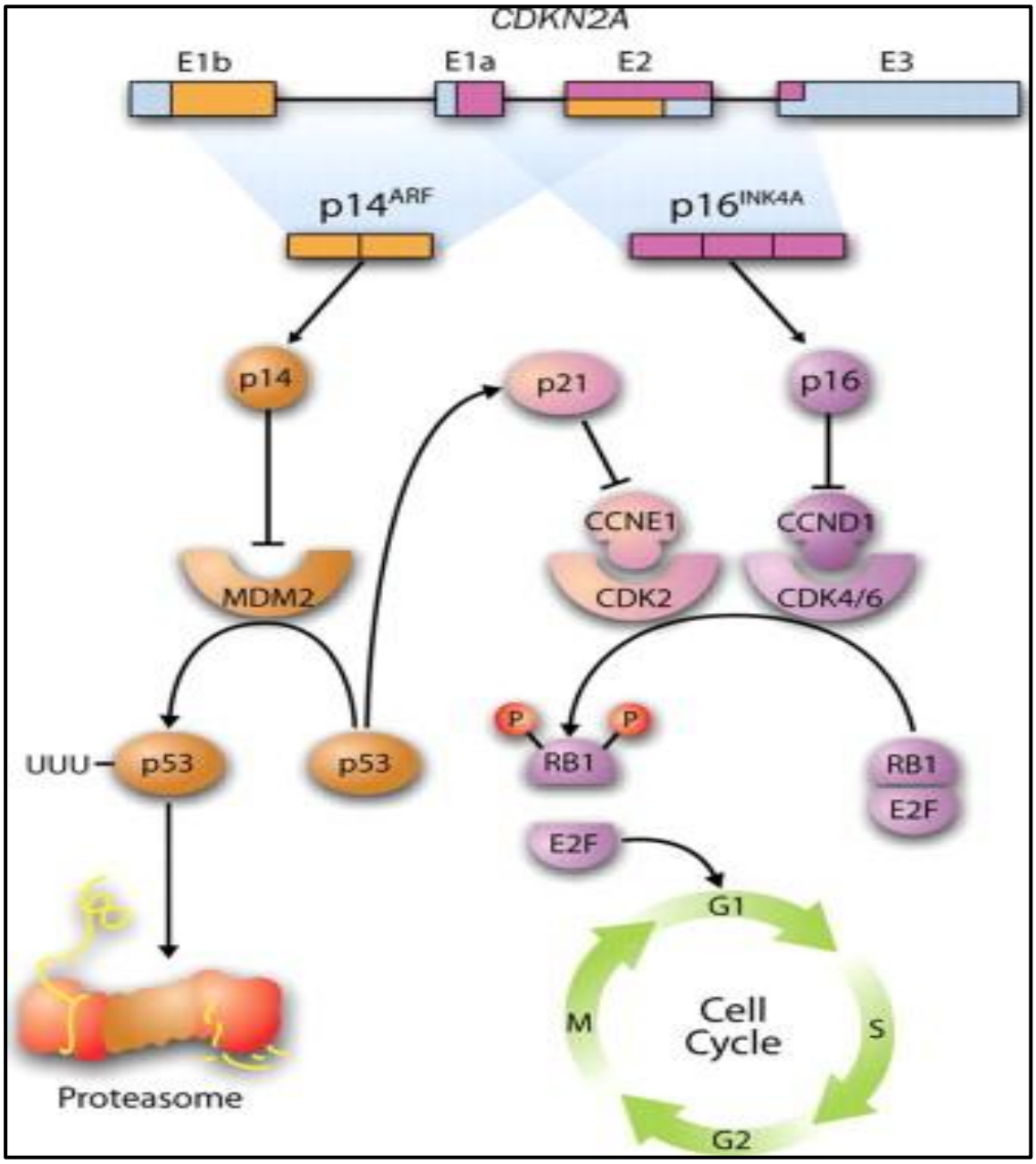
The dissertation topic titled "STUDY OF EXPRESSION OF p16 IN ORAL SQUAMOUS CELL CARCINOMA, POTENTIALLY MALIGNANT DISORDERS AND NORMAL MUCOSA" submitted by R.Sudharsan has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 26th September 2011.

Dr.K.Ranganathan
Secretary,
Ragas, IRB

Dr.S.Ramachandran
Chairman,
Ragas, IRB

ANNNEXURE - II

p16 PATHWAY:



ANNNEXURE - III

ABBREVIATIONS

1.	**OSCC**	–	Oral Squamous Cell Carcinoma
2.	**OSF**	–	Oral Submucous Fibrosis
3.	**HNSCC**	–	Head and Neck Squamous cell carcinoma
4.	**OPSCC**	–	OroPharyngeal Squamous Cell Carcinoma
5.	**WHO**	–	World Health Organisation
6.	**p53(or)TP53**	–	Protein 53 (or) Tumour Protein 53
7.	**pRb**	–	Phosphorylated Retinoblastoma gene
8.	**p16**	–	Tumour suppressor gene p16
9.	**p14**	–	Tumour suppressor gene p14
10.	**p15**	–	Tumour suppressor gene p15
11.	**bcl2**	–	B cell lymphoma 2 gene
12.	**Bax2**	–	B cell lymphoma associated X protein
13.	**CDK**	–	Cyclin Dependent kinases
14.	**CKI**	–	Cyclin Dependent Kinase Inhibitors
15.	**Rb**	–	Retinoblastoma gene
16.	**9p21**	–	p16 gene locus
17.	**CpG**	–	C - phosphate – G – Cytosine and Guanine nucleotide separated by phosphate
18.	**HPV**	–	Human Papilloma Virus
19.	**E6**	–	E6 Viral Oncoprotein
20.	**E7**	–	E7 Viral Oncoprotein
21.	**IHC**	–	Immunohistochemistry
22.	**H&E**	–	Hematoxylin and Eosin
23.	**LM**	–	Light Microscope

24. **APES** – Amino Propyl tri Ethoxy Silane

25. **HRP** – Horse Radish Peroxidase

26. **DAB** – Di Amino Benzidine

27. **DPX** – Di Butyl Phthalate in Xylene

28. **HCL** – HydroChloricAcid

29. **PBS** – Phosphate Buffer Saline

30. **INK** – Inhibitor Kinases

31. **Cip** – Cyclin dependent kinase Inhibitor Protein

32. **Kip** – Kinase Inhibitory Protein

33. **DNA** – Deoxyribo Nucleic Acid

34. **ARF** – Alternate Reading Frame

35. **E2F** – Transcription Factor E2F Family

36. **HeLA** – Henrietta Lacks cell line

37. **mRNA** – Messenger Ribo Nucleic Acid

38. cDNA **–** Complementary DNA

39. **p21** – Kinase Inhibitory Protein p21

40. **p27** – Kinase Inhibitory Protein p27

41. **PVL** – Proliferative Verrucuous Leukoplakia

42. **PCR** – Polymerase Chain Reaction

43. **PA28** – Proteasome Activator 28

44. **Ki 67** – Kiel 67 protein

45. **TNF** – Tumour Necrosis Factor

46. **IL** – InterLeukin

47. **TGF** – Transforming Growth Factor

48. **PAI** – Plasminogen Activator Inhibitor gene

49. **TIMP** – Tissue Inhibitor of Matrix Metalloproteinase gene

50. **INF** – Interferon

51. **FHIT** – Fragile Histidine Triad

52. **RB** – Retinoblastoma protein

53. **EGFR** – Epidermal Growth Factor

54. **RAS** – Rouse Avian Sarcoma

55. **EBV** – Epstein Barr Virus

56. **C(CAP)** – Head and Neck squamous cell carcinoma specific chromosomal alterations

57. **HN-CGAP** – Head and Neck cancer Genome Anatomy Project

58. **LCM** – Laser Capture Microdissection

59. **RASSF1A** – RAS associated domain containing protein

60. **PRAD1** – Parathyroid Adenomatosis 1 gene

61. **CYP1A1** – Cytochrome P450 family 1, Subfamily A, Polypeptide 1 gene

62. **GSTM1** – Glutathione S Transferase Mu1 gene

63. **MGMT** – Methyl Guanine methyl Transferase

64. **LMLH1** – L Mut L Homolog1 gene

65. **FISH** – Flourescent Insitu Hybridisation

66. **SSCP** – Single Stranded Conformational Polymorphism

67. **MTS1** – Multiple Tumour Suppressor 1 gene

68. **MDM2** – Murine Double Minute Protein

69. **CCNE1** – Conserved Cyclin E1

70. **CCND1** – Conserved Cyclin D1

71. **DAP-K** – Death Associated Protein Kinase

72. **LOH** – Loss Of Heterozygosity